U.PORTO

MESTRADO EM CIÊNCIAS DO MAR - RECURSOS MARINHOS

AQUACULTURA E PESCAS

Effects of dietary Amino acids supplementation on the European seabass (*Dicentrarchus labrax*) skin mucosal immune status.

Maria Carlota Pinto da Silva

M

2017

U.PORTO

Maria Carlota Pinto da Silva. Effects of dietary Amino acids supplementation on the European seabass (*Dicentrarchus labrax*) skin mucosal immune status.

Effects of dietary Amino acids supplementation on the European seabass (*Dicentrarchus labrax*) skin mucosal immune status.

Maria Carlota Pinto da Silva

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

M.ICBAS **2017**

MARIA CARLOTA PINTO DA SILVA

EFFECTS OF DIETARY AMINO ACIDS SUPPLEMENTATION ON THE EUROPEAN SEABASS (*Dicentrarchus labrax*) SKIN MUCOSAL IMMUNE STATUS

Dissertação de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Doutor Benjamín Costas
Categoria – Investigador Auxiliar
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental

Co-Orientador – Professor António Afonso
Categoria – Professor Associado
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Agradecimentos

This work was supported by Project ALISSA (reference ALG-01-0247-FEDER-3520), financed by Portugal and the European Union through FEDER, COMPETE 2020 and CRESC Algarve 2020, in the framework of Portugal 2020.

Este foi um ano de muita aprendizagem. Agradeço em especial ao Professor Afonso pela grande oportunidade de me ter integrado no seu grupo de investigação. A sua personalidade absolutamente cativante fez-me procurá-lo na escolha do tema desta tese.

Agradeço imenso ao Professor Benjamín por ter sido o meu orientador. Muito obrigada pelas suas correções, conselhos e por ter criado uma excelente oportunidade para mim.

Professor Eduardo! Foi tão importante! Todo o seu brilho e entusiasmo quer dentro da sala de aula, quer numa conversa acidental nos corredores do ICBAS, inspiraram-me verdadeiramente. Obrigada!

O melhor que esta tese me trouxe foi fazer parte da equipa do laboratório NUTRIMU, sem a qual este trabalho não seria possível! Aprender e trabalhar todos os dias com este verdadeiro espírito de equipa vai deixar muitas saudades! Cada um à sua maneira, contribuiu para a realização deste trabalho e, por isso, deixo aqui um grande abraço de agradecimento a todos: Rita Azeredo, Filipa Fontinha, Francisco Guardiola, Joana Moura, Paulo e Renata. Obrigada ao meu ilustre colega Lourenço, pelas gargalhas diárias até sermos separados, pelos truques no computador e explicações durante os meus pânicos estatísticos (“não estou mesmo a perceber”). A verdade é que grandes progressos nesta tese foram feitos quando estavas à minha frente na secretária ou ao meu lado na bancada do laboratório. Obrigada amigo! Mr. Bruno, obrigada! Para além do teu raciocino químico, que interveio muitas vezes nas minhas contas e quando os reagentes não dissolviam, quero agradecer-te pelos teus conselhos dentro do laboratório e por todas as risotas na varanda lá de cima. Serginho, muito obrigada por todo o cuidado e preocupação que mostraste desde o meu primeiro protocolo (e pelas tuas colheitas biológicas deliciosas também)! Mas o maior obrigado de todos, é para a Marina!

Estiveste presente em todos os protocolos e P-values, na escolha das cores dos meus gráficos, até à última frase da conclusão! Muito muito obrigada chefe, por tudo o que me ensinaste e pela tua infinita paciência, principalmente nos momentos que eu levei mais desassossego para laboratório e para os dias de amostragem! Foste essencial e obrigatória, por isso, espero mesmo que gostes desta tese, porque ela também é um bocadinho tua!
Amigaço Tiago, foste tão tão importante!!! Começamos e acabamos este curso juntos e não há companheirismo como o nosso! Obrigada!

Pai, Mãe... nem sei bem o que dizer! Com dois heróis como os pais, sempre do meu lado a orientar-me, tem sido fácil. Mãe, obrigada pela tua preocupação constante de mãe, e por me fazeres ver, sempre, as oportunidades da maneira mais realista! Pai, obrigada por me salvares de todos os meus contratempos (que são muitos), por me apoiares incondicionalmente em todos os meus sonhos, até os mais impossíveis, e por festejares as minhas conquistas como se fossem as tuas! Irmãos, queridos e irritantes ao mesmo tempo, obrigada por me fazerem lembrar de tudo, menos da tese!

Minha querida Baba! És a melhor Avó do mundo e os teus mimos culinários apareceram sempre nos momentos em que eu mais precisei de inspiração! Mas muito mais importante, obrigada pelo orgulho que sempre mostraste pela tua neta, mesmo quando não percebias exatamente o que eu estava a estudar e querias contar às tuas amigas.

Agradeço profundamente às minhas amigas, que estão e estiveram sempre bem próximas do meu metro quadrado. Esta tese reflete, com certeza, a vossa amizade permanente. Muitíssimo obrigada Mimi, Mariana Sá, Margarida e Joana Simões, Inês Figueiredo, Mariana Veiga e Kika! Fil, Vaninha e Dudu, que quase marcaram voo antes de mim durante as minhas falsas partidas para as Canárias, obrigada pela vossa imensa vontade me ir visitar. Isto sim, é amizade!!!!! Pelas abundantes visitas refundidas no Jardim Botânico e pelas minhas desorganizações mais profundas em Hollywood, pela intensa comunicação Madrid-Porto... foste e és indispensável, Sofia Freitas. Obrigada Inês e Matilde pela vossa presença assídua e pontual nos finais de tarde da Associação a seguir ao trabalho. Deram-me muita forcinha para o dia seguinte. Às minhas queridas e adoradas amigas Viky e Marta, que mesmo

estando em Espanha, e os meus aparelhos de comunicação sempre estragados, nunca desistiram de arranjar outras alternativas de me procurar.

Importantíssimo, obrigada Tiago PL! Embora tenhas reduzido significativamente os meus níveis de concentração e de produtividade, a tua gasolina de avião motivou-me todos os dias!

Resumo

Os maiores custos económicos na indústria de aquicultura devem-se essencialmente às despesas relativas à alimentação dos animais e às perdas de stock causadas por doenças infeciosas. O foco da investigação no âmbito da aquicultura tem vindo a ser o desenvolvimento de dietas funcionais de forma a evitar o aparecimento de doenças e, consequentemente, o seu tratamento. Os aminoácidos (AA) são considerados importantes reguladores de vias metabólicas, crucias para a resposta imune e, por este motivo, representam potenciais candidatos para integrar estas dietas.

Este estudo teve como objetivo investigar os efeitos da suplementação nutricional de metionina, da arginina e da citrulina no estado imune do robalo (*Dicentrarchus labrax*). Os efeitos da dieta foram avaliados pela medição dos parâmetros imunes do muco da pele. A hipótese experimental levada a cabo neste trabalho, visa verificar se estas dietas podem modular o estado imune do muco da pele do robalo, quer na ausência de doenças, quer em situação de infeção bacteriana.

Foram realizados dois ensaios separadamente, mas de desenho experimental semelhante, com o intuito de descobrir de que forma cada um dos AA influencia os parâmetros imunes do muco do robalo. O ensaio 1 foi focado no impacto da metionina. Para tal, foram formuladas duas dietas com suplementação de 0.5% e 1% de metionina, uma terceira dieta controlo cujos níveis de metionina estavam de acordo com necessidades nutricionais estabelecidas para robalo e ainda uma quarta dieta deficiente em metionina.

No ensaio 2 foi avaliada a influência da arginina e da citrulina na imunidade do muco. Assim, foi formulada uma dieta controlo que cumpria com os requisitos de arginina e de citrulina estabelecidos para o robalo e três outras dietas suplementadas com 1% e 2% de arginina e a terceira com 1% de citrulina.

Em ambos os ensaios (1 e 2), os peixes foram alimentados com as dietas experimentais durante 4 semanas e foram realizadas amostragens de muco às 2 e 4 semanas. No final do período de alimentação, os peixes foram submetidos a uma infeção bacteriana por injeção peritoneal com *Photobacterium damselae* subsp. *piscicida* (*Phdp*). No ensaio 1, os peixes foram amostrados às 4 e 24 horas após a injeção, enquanto que no ensaio 2 os peixes foram amostrados às 4, 24 e 48 horas após a injeção da bactéria. Os parâmetros humorais do muco da pele do robalo

foram estudados durante o período de alimentação (duas e quatro semanas),e nas horas seguintes à infeção, nos dois ensaios.

Relativamente à suplementação de metionina, demonstrou-se algum grau de influência na resposta do muco ´á inflamação no peritoneu. Foi verificado o decréscimo dos níveis de lisozima e peroxidase no muco, provavelmente devido ao recrutamento de leucócitos para o local de infeção.

A arginina parece exercer efeitos benéficos sobre o estado imune do muco do robalo, uma vez que foi observada uma tendência de aumento da atividade bactericida e peroxidase em todos os grupos suplementados. Quando a bactéria foi injetada na cavidade peritoneal, o reforço da imunidade observado, traduziu-se numa diminuição dos parâmetros humorais do muco. Em conjunto, estes resultados sugerem que, a arginina estimulou o desvio da energia metabólica dos tecidos periféricos para o foco inflamatório. Acresce ainda que a arginina permitiu a recuperação dos níveis de lisozima, possivelmente devido à proliferação de macrófagos na pele, nas 48 horas após a injeção.

Conclui-se, com este estudo que a suplementação com arginina, metionina ou citrulina na dieta do robalo, não tem uma ação pronunciada e direta sobre a resposta imune do muco após inflamação peritoneal. No entanto, os resultados sugerem uma possível migração celular do tecido periférico para o foco inflamatório, promovida pelos AA estudados.

Palavra-chave: Robalo; Imuno-modulação; inflamação; muco da pele; parâmetros humorais; aminoácidos; *Photobacterium damselae* subsp *piscicida*

Abstract

Major expenses in aquaculture industry are probably attributed to infectious diseases and fish feeds management. Recently, much attention has been given to the use of nutritional strategies for preventive health care, aiming to avoid the occurrence of diseases and its therapeutic procedures. Emerging evidence shows that many amino acids (AA) regulate key metabolic pathways that are crucial to immune responses, making them good candidates to be included in functional feeds.

Having this in mind, the present study aimed to investigate the effects of dietary methionine, arginine and citruline supplementation on the European seabass (*Dicentrarchus labrax*) immune status. The dietary treatments were evaluated by measuring skin mucus immune parameters. Our experimental hypothesis was to assess whether those dietary treatments can modulate the skin mucus health status. Two trials, with a similar experimental design were performed separately. Trail 1 was focused on dietary methionine surplus, and thus, two diets with 0.5% and 1% methionine were formulated; plus, a control diet whose methionine levels met the requirement for seabass; and a fourth diet deficient in methionine. In trial 2, arginine and citruline influence on mucus immune parameters was evaluated. Three supplemented diets (1% and 2% arginine supplementation; 1% citrulline supplementation) and a control diet (meeting arginine and citruline requirement levels for seabass) were formulated. In both trials, fish were fed with experimental diets for 4 weeks, and mucus sampling was performed after 2 and 4 weeks of feeding. At the end of the feeding period, fish were subjected to a bacterial infection by intraperitoneally injecting *Photobacterium damselae* subsp. *piscicida* (*Phdp*) strain PP3. Fish were sampled following 4 and 24h post-injection in trial 1; whereas in trial 2, three samplings were performed after 4, 24 and 48h post-injection. For both trials mucus humoral parameters were studied during the feeding period, and during the hours following the infection.

Methionine showed some degree of influence in mucus immune response. After bacterial infection, methionine supplemented groups showed a decrease in lysozyme and peroxidase levels, probably attributed to the recruitment of immune cells towards infection site.

Arginine appears to exert beneficial effects on unchallenged European seabass immune status, since a trend to augment bactericidal and peroxidase activities was

detected in the skin mucus of fish fed supplemented diets. Interestingly, when the bacterium was injected in the peritoneal cavity, the enhanced immunity observed, translated in a decline of mucus humoral parameters. Together, these results suggest that, upon bacteria stimuli, there is an enhanced deviation of immune energy from peripheral tissues to the inflammatory focus fueled by arginine. Moreover, in the particular case of lysozyme activity, arginine allowed a recovery of the depressed levels following injection, possibly due to macrophages proliferation.

In summary, it is suggested that neither arginine nor methionine supplementation have a pronounced and direct influence on the mucus innate immune response to a peritoneal inflammation. However, the results give us insights of a possible cellular migration from the peripheral tissue towards inflammatory focus, fostered by the studied AA.

Key-words: European seabass; immunomodulation; inflammation; skin mucus; humoral parameters; amino acids; *Photobacterium damselae* subsp. *piscicida*

Index

List of figures and tables

1.

List of Abbreviations

1. AA – Amino acids;
2. Phdp - Photobacterium damselae subsp. piscicida strain PP3;
3. MALT - Mucosa-associated lymphoid tissue;
4. GALT - Gut-associated lymphoid tissue;
5. SALT - Skin-associated lymphoid tissue;
6. GIALT - Gill-associated lymphoid tissue;
7. NALT - Nasopharynx-associated lymphoid tissue;
8. SAM - Decarboxylated S-adenosylmethionine;
9. iNOS - Inducible nitric oxide synthase;
10. NO – Nitric oxide;
11. ACP - Alternative complement pathway;
12. CTRL – Control diet;
13. MET0.5 - Diet supplemented with DL-Methionine at 0.5%;
14. MET 1. - Diet supplemented with DL-Methionine at 01%;
15. NCRT – Diet deficient of methionine;
16. i.p. – Intraperitoneally;
17. ARG1 - Diet supplemented with DL-Arginine at 0.5%;
18. CIT1 – Diet supplemented with DL-citrulline at 0.5 %;
19. ARG2 - Diet supplemented with DL-Arginine at 1%;
20. SD – Standard Deviation;

Introduction

Aquaculture

Aquaculture has gain great deal of importance, over the last few decades, with a production of 73.8 million tonnes in 2014, valued at US$160.2 billion. In 2014, aquaculture supplied 44.1% of the total fish consumed by humans (Fig.1). This number is expected to increase in the next times, as all continents apart from Oceania show a clear tendency to intensify their aquaculture production. However, China is the major responsible for this growth, contributing with around 60% of Global fish production from aquaculture, a remarkable value compared with the European Union´s little contribution (4%) (Fig.2).

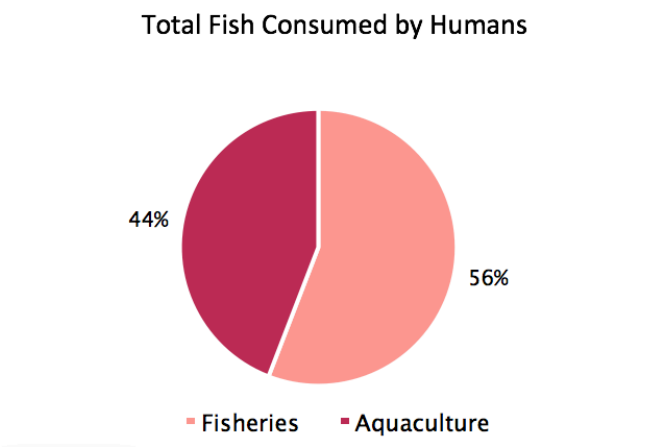


Figure 1 *- Total Fish Consumed by Humans in 2014.Adapted from:* (FAO, 2016)

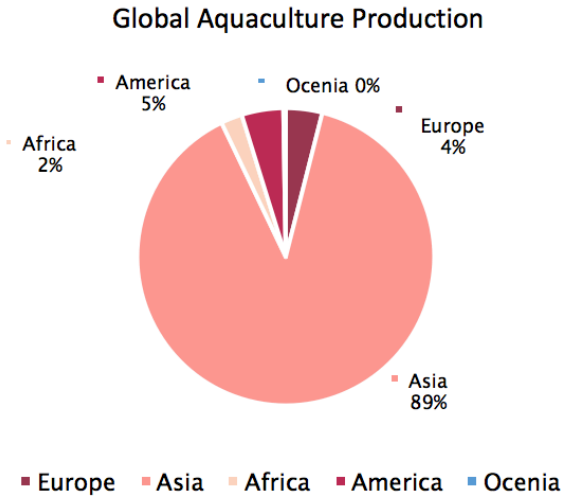


Figure 2*- Global Aquaculture Production. Adapted from:* (FAO, 2016)

In the last decades, the animal protein intake has raised immensely driven by the population growth, urbanization and economic development. Fish and its derivates may have two distinct origins: either captured by wild fisheries or farmed in aquaculture. Aquaculture hold great promise for meeting global demand for fish supply, as capture fishery production is no longer a solution. Besides the fact that fisheries are static since 1980, nowadays, about 31.4 % of fish stocks were estimated as fished at a biologically unsustainable level.

In developing countries, the annual per capita consumption of fish is still lower (18.8 kg in 2013) to that of developed regions (26.8 kg in 2013). Yet, this discrepancy tends to be lower over the time, as fish intake in developing countries had an impressive growth since 1961. At the moment, Europe's fish production is not enough to satisfy the population demand and imports are inevitable. As the world major producers; China, India, Vietnam, Bangladesh and Egypt provide a sizeable share of fish to be imported. An increased of fish consumption in developing countries will negatively affect the Europe´s trading balance, once there will not be sufficient fish production to cope with both exportations and national feeding. Thus, imports are likely to become scarcer and more expensive, given the growing purchasing power on China and Asiatic countries. The solution would be to augment the national aquaculture sector in developed nations, to keep up with domestic demand, ensuring a sustainable and independent fish market (FAO, 2016).

Portugal despite being one of countries with high seafood consumption rates, has a troubling seafood trade deficit. Until 1986, national aquaculture was low and mainly characterized by the bivalves and freshwater trout. With the integration in EU, new technological improvements were implemented fostered by EU incentives, resulting in the growth of national production (Lopes, 2016). However, from 1988 to 2011, a gradual decline on aquaculture production was observed, and seafood consumption was sustained by increasing dependency in external markets (Ramalho & Dinis, 2011). According to the Portuguese Association of Fish Farmers in 2011 Portuguese aquaculture totalled 9,000 tonnes, corresponding to around 1.6% of the total national seafood consumption. Yet this sector is expected to grow during the next years supported by European Union funding. Moreover, Portuguese aquaculture will benefit from the extension of Portugal continental shelf beyond 200 nautical miles, as new territory with economical interest will be available. Portugal exhibits ideal conditions for the offshore aquaculture, offering one of the

largest coast lines in Europe associated to productive waters, sustainable temperature range. Despite the promising geographic location, aquaculture development in Portugal has a number of constraints (Ramalho et al., 2011). The difficulties faced by the aquaculture industry in Portugal are mostly due to bureaucratic impediments with complex licensing procedures and the large number of governmental institutions involved. Additionally, Portugal lacks on coastal management planning, attractive market prices, which complicates and delays the progress of Portuguese aquaculture and discourages the investment on this sector (Lopes, 2016).

Generally, the aquaculture production worldwide is impaired by various factors such as diseases, lack of fishmeal alternatives, environmental conditions, scientific and technological limitations, among others. Aquaculture intensification implies maximum culture densities, which compromise the animal welfare and favors the emergency of diseases. Thus, there is an urgent need for effective disease control measures that may allow greatest productivity with minimum occurrence of infectious episodes. Another major constraint to the production process is fish nutrition. Fishmeal is the main constituent of fist diet, representing the most ideal protein source for fish growth. However, due to its static global production, fishmeal price is high, with limited availability. Thus, aquaculture is now facing a difficult challenge of replacing fishmeal and fish oil by more sustainable protein sources. The scenario is worsened by the economic instability and climate changes observed nowadays (FAO, 2016).

European Seabass Dicentrarchus labrax

The European seabass *Dicentrarchus labrax* (Linnaeus, 1758) (Fig.3) is common all over the Mediterranean Sea, the Black Sea and along the North Eastern Atlantic coasts, from Norway to Senegal. Being a eurythermic and euryhaline species, it is able to survive in both fresh and high salinity waters (i.e. 3‰ to full strength sea water); and tolerate a wide range of temperatures from 2 to 32˚C. They are found in estuarine areas, and coastal lagoons during summer, but migrate to offshore waters in the winter (Moretti, Fernadez-Criado, Cittolin, & Guidastri, 1999).

Sexual maturity generally occurs at 3 years in males and at 4 years in females in the Mediterranean Sea, whereas in the Atlantic Ocean it occurs at 4 and 7 years,

respectively. Though, under farming conditions puberty is achieved at 2 and 3 years for male (± 200 g) and female (± 700 g). There is only one spawning season per year, from December to March, which takes place in estuaries and in-shore areas, where the salinity is high, and temperature is at 12-14ºC. European seabass spawn 492 000 to 950 000 eggs/ Kg body weight in the Mediterranean Sea, and the eggs are small, pelagic and start hatching more approximately 27h after fecundation.

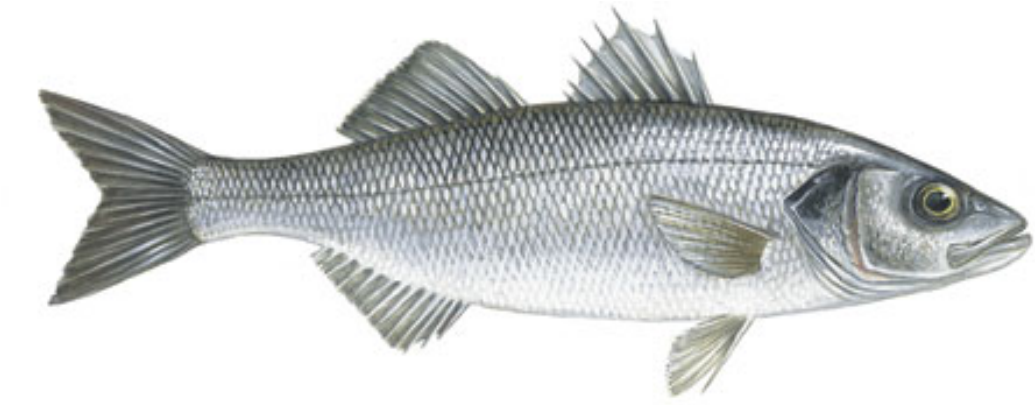


***Figure 3**- European seabass Dicentrarchus labrax. Source: (FAO, 2012)*

In the last decade, seabass industry has grown immensely in Europe, particularly in Mediterraean areas. Nowadays, it is considered to be one of the most important commercial fish in EU, with Greece, Turkey, Italy, Spain, Croatia and Egypt being the biggest producers. Farming may take place in extensive, semi-intensive and intensive systems; and farm operations may integrate all stages of production cycle: egg production, larvae rearing, weaning, juvenile production (or pre-ongrowing) and ongrowing. According to (Basurco, 2000), ongrowing seasbass is farmed in most cases in net cages (intensive regime). Other farms raise seabass in land-based methods, such as flow-through and recirculation system.

Aquaculture Constrains

Economic losses in aquaculture are mainly due to infectious diseases and its non-effective treatments such as approved antibiotics and chemotherapeutants (Kiron, 2012). Besides not being eco/consumer-friendly, the treatments often do not show a beneficial effect on the fish health (Pohlenz & Gatlin, 2014). However, aquaculture creates favorable conditions to the emergency of diseases.

An intensive aquaculture production is unavoidably a stressful environment for fish to grow, due to high fish densities at minimum space. Indeed, chronic stress is known to impair the immune system and immunosuppressed fish display fewer defences towards a bacterial insult (Conceição et al., 2012). Moreover, under intensive aquaculture practices, the first line of defence of fish (e.g. mucus, epidermis, and scales) is often compromised due to physical abrasion, providing easy access for pathogens. Thus, the is a need to improve the health of fish's mucosal surface, for instance by the implementation of good management practices, which in turn will result in less host susceptibility to any invading agent. Particularly when handling the fish, farmers must be concern with the maintenance of fish's mucosal integrity.

One of the most threatening bacterial diseases, affecting mainly Mediterranean aquaculture, is the Photobacteriosis (formerly pasteurellosis). The infection is caused by *Photobacterium damselae* subsp. *piscicida* , a gram-negative, halophilic bacterium (Magarinos, Toranzo, & Romalde, 1997). Generally, Photobacteriosis does not provoke surface lesions, thus no major external alteration of the infected fish is perceptible. On the other hand, internal pathological changes may vary depending on whether the disease is chronic or acute (do Vale et al., 2007). It is recognized to cause massive mortalities due to its 1) great resistance to antibiotics; 2) ability to infect more than 20 hosts; 3) widespread geographic distribution; 4) and the lack of efficient vaccines (Do Vale et al., 2005). Its success as a highly pathogenic agent, relies on the strategy to avoid the host's phagocytic mechanism. Do Vale, Marques, & Silva (2003), reported that virulent *Phdp* strains secrete AIP56, an exotoxin, which induces extensive apoptosis of host's macrophages and neutrophils. For these reasons, *Phdp* is known to cause a lethal septicemia in species such as Senegalese sole (*Solea senegalensis*), gilthead seabream (*Sparus*

aurata) and European seabass, being responsible for huge economic losses in Mediterranean farms (Romalde, 2002).

Mucosa

In contrast to terrestrial animals, fish are in constant interaction with a microbial-rich environment present in the water that circulates through their body. Thus, every epithelial barrier of fish is continuously challenged with high microbial loads, specially in an aquaculture context (Subramanian, MacKinnon, & Ross, 2007). To cope with these aggressions, fish develop mucosal surfaces as a defense mechanism from the external environment. Besides being a physical boundary, fish mucosal surfaces represent a metabolic active tissue armed with both humoral and cellular immune components. The mucosa-associated lymphoid tissue (MALT) is considered to be a secondary lymphoid organ due to its ontogeny and functional characteristics. According to anatomical location, the MALT in teleost fish is subdivided in gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT) and the recently discovered nasopharynx-associated lymphoid tissue (NALT) (Fig.4). Since the majority of the infectious agents affects or initiates the process of infection in the mucous surfaces, the mucosal immune response plays a crucial role in the course of the infection

Both adaptive and innate immune responses have been reported in each of those mucosal body surfaces (Salinas, 2015). Moreover, the MALT is specialized to tolerate a diverse microbial community. These tolerance is key for the homeostasis maintenance, otherwise a useless immune response would be constantly mounted against non-harmful antigens. However the modulation of MALT by this microbiota is largely unknown (Salinas, Zhang, & Sunyer, 2011).

In aquaculture industries, mucosa health of fish should be given prime importance as it constitute the first line of defense against highly stressful conditions. A better understanding of mucosal immune systems should be generated, which in turn, will help to develop treatment strategies.

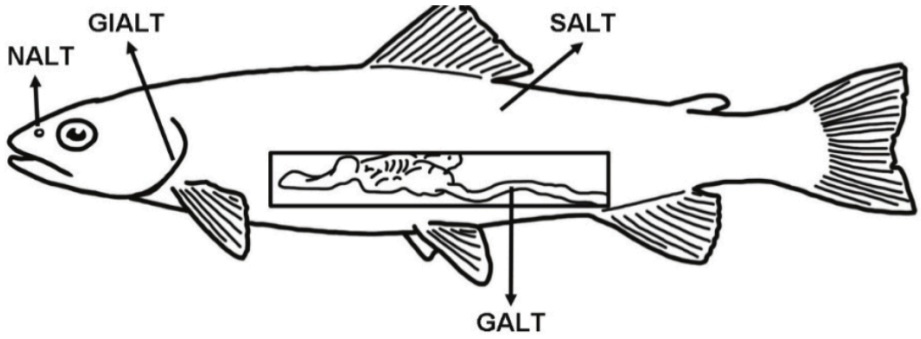


***Figure 4**- Schematic representation of the four teleost main mucosa-associated lymphoid tissues (MALT) described so far and their anatomical localization. GALT: gut-associated lymphoid tissue; SALT: skin-associated lymphoid tissue; GIALT: gill-associated lymphoid tissue; NALT: nasopharynx-associated lymphoid tissue. Source: (Salinas, 2015)*

SALT- Skin associated lymphoid tissue

Teleost skin is a large, unique and histological diverse organ. The skin is the envelope that separates the fish from its environment and it is constitute by three layers: the mucus (outermost epidermal barrier, with complex composition); epidermis (a squamous stratified epithelium); dermis (with two layers, the hypodermis or stratum spongiosum, and the innermost layer or stratum compactum) (Ángeles Esteban, 2012). In particular, teleost epidermis is very different to that of mammals because it secretes mucus which is involved in immune functions (Ángeles Esteban, 2012). The skin associated lymphoid tissue (SALT) correspond to all immunological defenses provided by fish skin (Ángeles Esteban, 2012). Unlike mammals, the teleost skin is not keratinized and thus the epithelium cells are alive and retain the ability to divide (Salinas et al., 2011). Therefore, the skin epithelium represents an active immune site, coated by mucosa layer which is produced by four types of secretory cells: goblet cells, sacciform cells, Malpighian cells and club cells (Zaccone, Kapoor, Fasulo, & Ainis, 2001). The mucus, besides harboring important immune component has also been reported to have a key functions including respiration, ionic and osmotic regulation, reproduction, communication, excretion, feeding and next building (Shephard, 1994).

The mucus provides both mechanical and chemical protection to fish. As a component of the innate and adaptive immune mechanism, it harbors a great variety of antimicrobial substances include, lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein, proteolytic enzymes and antibacterial peptides and proteins (Shephard, 1994). Moreover, as it is constantly produced, it prevents the adherence and colonization of parasite, bacteria and fungi on the fish skin (Pickering, 1974). Although very little information is available, the main cellular immune constituents of teleost SALT are lymphocytes, granulocytes, macrophages, and Langerhans-like cells (Salinas et al., 2011). Moreover, as a component of innate and adaptive immune mechanisms, it harbors a great variety of antimicrobial substances such us lysozyme, immunoglobulins, complement proteins, lectins, C-reactive protein, proteolytic enzymes and antibacterial peptides and proteins (Shephard, 1994).

Hence, as part of a primary defence mechanism of epithelia mucosa, the skin mucus is expected to adapt itself to pathogenic pressure and external variations. Indeed, if SALT could accurately discriminate between fish exposed to a pathogenic agent from healthy ones, mucus sampling methods may be employed during farming procedures to predict and assist on future disease management. This technique may constitute a valuable vantage for farmers, to better understand some abnormal behavior, for instance, without requiring invasive methods.

Amino acids (AA) needs under aquaculture conditions

An acute infection is characterized by a sharp depletion of AA availability, which are diverted from normal metabolism to be consumed as substrate for the immune response and synthesis of stress and immune-related proteins (Conceição et al., 2012). Prolonged infections are also associated with AA imbalances, weight losses caused by net protein breakdown. Similarly, under stressful conditions, fish AA requirements increase to cope with the augmented metabolism and energy demands. The restoration of the AA pool will avoid the lack of recourses, allowing the fish to cope with increased energy demands. This is the basis of Immunonutrition concept.

Immunonutrition

Immunonutrition aims to provide the right molecules to support immune responses through fish diet. An additional nutrients supply may assist the immune system to realize its functions, to finally obtain a higher degree of protection (Kiron, 2012). An efficient immune defence is of the upmost importance in farms, once fish are constantly exposed to both stress factors and pathogen invasion (Fig.5).

Moreover, it is widely recognized that the development of aquaculture industry should be based in eco-friendly and sustainable managements. Thus, the use of nutritional means for preventive health care had already proved to be a potential strategy to achieve sustainability in aquaculture. Instead of fighting a certain disease by using vaccines or antibiotics, immunonutrition aims to prevent pathogens invasion. For instance, with an increased AA availability, the fish would be better prepared to mount a proper immune response. However, the link between nutrition, immune responses and resistance to diseases needs to be clearly understood, to access the dietary manipulation impact on fish growth and survival.

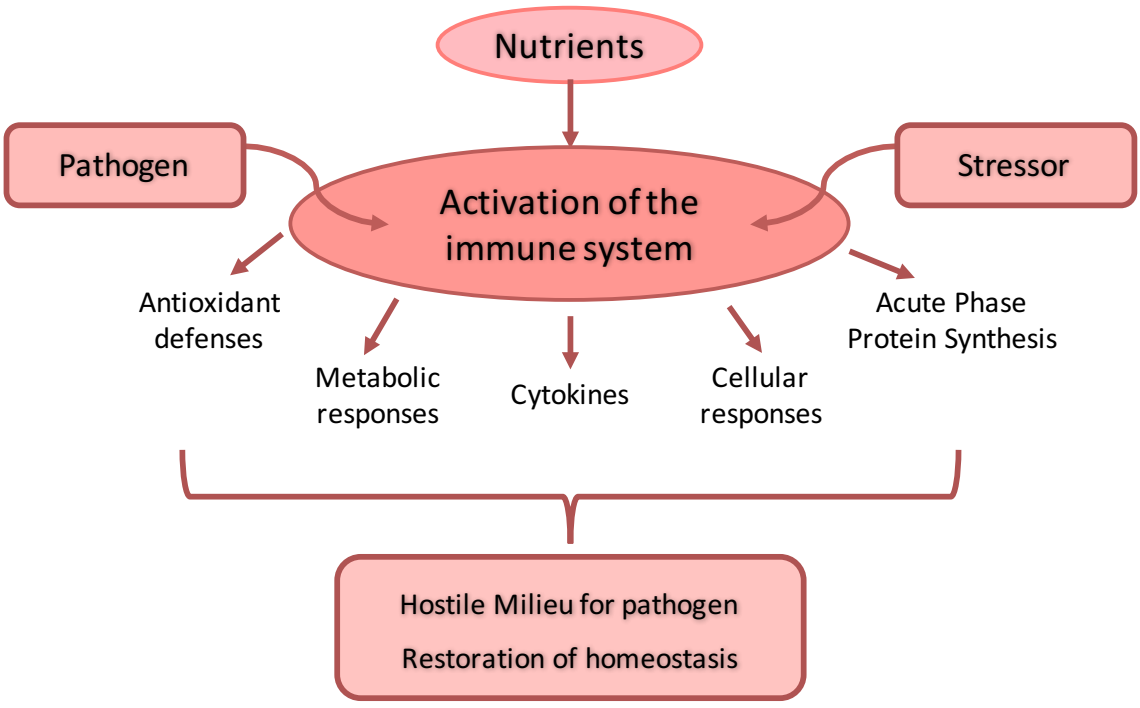


***Figure 5**- Immunonutrition Concept. Adapted from (Kiron, 2012).*

Methionine

Indeed, AA have a central role in the defense mechanism since they are involved in the synthesis of important molecules. Methionine is an essential AA, and it is usually a limiting one in fish diets with high inclusion of plant protein sources (Wang, Qiao, & Li, 2009). Indeed, this AA is the first limiting step in the initiation of protein synthesis and proteolytic pathways. Methionine has at least four main metabolic pathways known for mammals to affect the immune system, which are likely to be present in fish (Rubin et al., 2007). For instance, methionine is involved in T cell proliferation and differentiation by fuelling polyamines biosynthesis (spermine and spermidine) (Fig.6). Moreover, through the generation of decarboxylated S-adenosylmethionine (SAM), methionine is the most important methyl donor for methylations reactions of DNA and proteins (Fig.7). Finally, methionine is also a glutathione precursor (Métayer et al., 2008).

Glutathione affects the immune system either by protecting the body from oxidative stress, but also in supporting T-cell Proliferation. Glutathione is a key antioxidant molecule, capable of reducing free radicals and ROS during an inflammatory response (Grimble, Grimble, Poshoi, al., & Lauterberg, 1996). Moreover, T-lymphocytes and polymorphonuclear leucocytes are sensitive to intracellular glutathione concentrations (Machado et al., 2015).

In fact, dietary methionine supplementation appears to exert clear positive effects on the European seabass (*Dicentrarchus labrax*) immune status by improving the peripheral leucocyte response followed by higher complement activity and bactericidal capacity in response to inflammatory insult with inactivated *Phdp* (Machado et al., 2015).

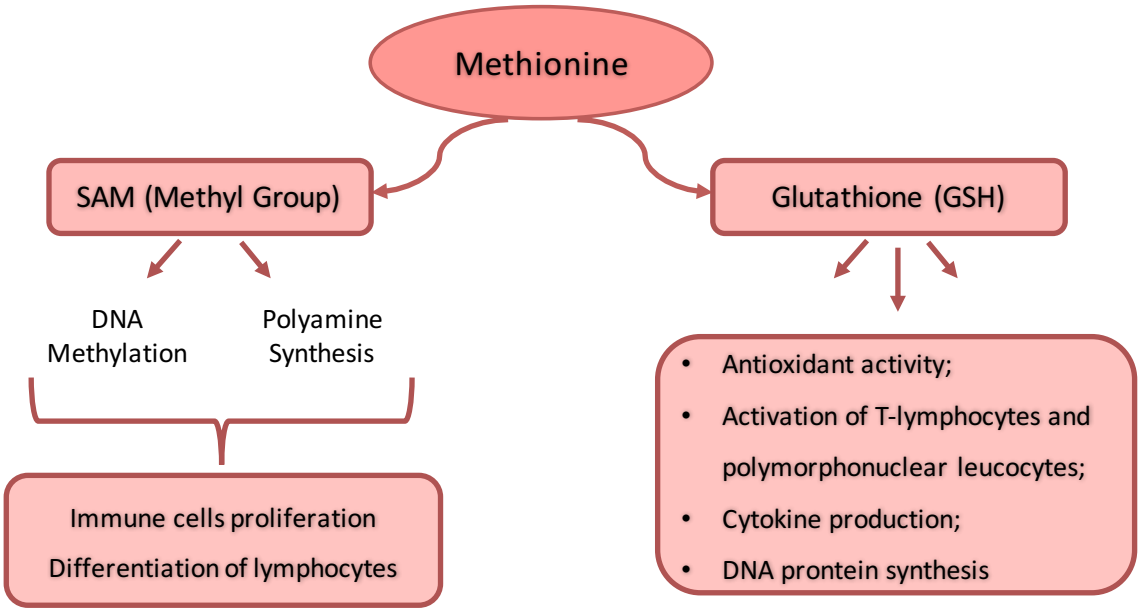


Figure 6- Methionine in immune function.

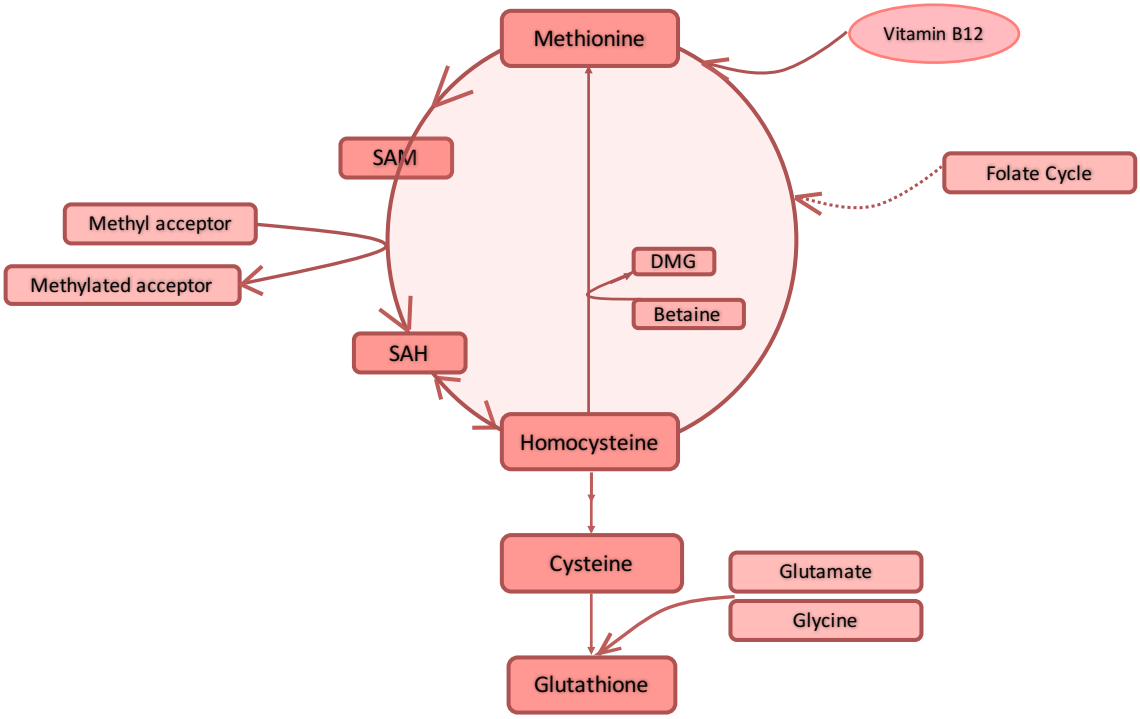


***Figure 7** – Methionine metabolism. SAM-decarboxylated S-adenosylmethionine; SAH- S-adenosylhomocysteine*

Arginine

Arginine is a versatile essential AA for all fish species investigated to date, taking part in several metabolic reactions (Conceição et al., 2012). Besides being an abundant constituent of body proteins, arginine is also present in the tissue fluids as phosphoarginine (Li, Mai, Trushenski, & Wu, 2009). There is no knowledge regarding the synthesis of arginine from another AA apart from citrulline. For these reasons, arginine *de novo* synthesis is very limited or complete absent in some fishes, and may be particularly compromised during stress events. Under unhealthy conditions, arginine levels may not be enough neither to support optimal growth or metabolic process.

Similarly to terrestrial animals, arginine plasma levels in fish are controlled by two main catabolitic enzymes, the inducible nitric oxide synthase (iNOS) and arginase. The later converts arginine into ornithine and urea, whereas iNOS oxidizes arginine in two steps that generate nitric oxide (NO) and citrulline. (Conceição et al., 2012) (Fig. 8).

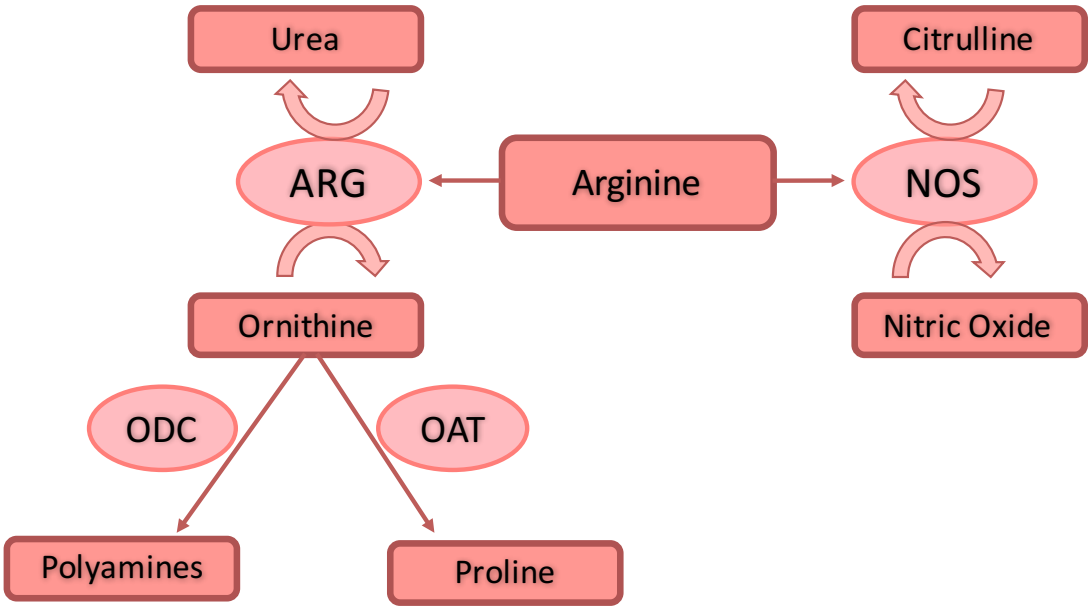


***Figure 8**– Arginine metabolism. ARG-arginase. NOS-nitric oxide synthase. ODC-ornithine descarboxylase. OAT-ornithine aminotransferase. Adapted from* (Wu et al., 2009)

Both metabolic pathways are critical to the immunomodulatory actions of arginine. They directly enhance the immune system responses through 1) polyamine synthesis and; 2) NO production.

Polyamines are positively related to RNA and DNA polymerase activity and appear to be indispensable in cell division, DNA replication and regulation of the cell cycle (Li, Yin, Li, Kim, & Wu, 2007). Besides arginase pathway, arginine can also augment polyamines synthesis through the stimulation of the release of growth hormone (Nieves & Langkamp-Henken, 2002).

NO is a powerful oxidant and an anti-microbial agent against parasites, fungi, bacteria and viruses (J. a Buentello & Gatlin Iii, 1999). In fact, phagocytic efficiency is closely related to nitrogen reactive species in fish (G. Chen et al., 2015). Upon infection, NO acts increasing the environmental toxicity, which will compromise the integrity of the structures of both host and pathogen (Cheng, Buentello, & Gatlin, 2011).

However, although emerging evidences supporting the arginine promoting effects on growth, immunity or resistance to environmental stressors and pathogens, some authors confirmed that fish fed with diet containing excessive arginine shown immune-depressed symptoms (Ren et al., 2013) (Xie et al., 2012) (Azeredo et al., 2015).

Arginine is able to mediate immunosuppressive pathways. Jiang et al., 2015 reported that LPS-induced inflammatory response was inhibited when arginine was added to both primary enterocyte culture media and to the diet of Jian carp (*Cyprinus carpio var. Jian*).

In contrast, arginine promoted the synthesis of immune-related proteins and compounds such as cytokines, complement, lysozyme and antibodies during bacterial exposure. Moreover, lymphocyte proliferation and differentiation was also observed upon arginine surplus, as well as increased survival rates after bacteria exposure (G. Chen et al., 2015). Some of the results are resumed in table 1.

Table 1 *- Summary of fish studies showing the effect of arginine on various immune parameters.*

Species	Model	Arginine Supplementation	Effect on immune parameters		Reference
			Effect	No effect	
Channel catfish (*Ictalurus punctatus*)	In vitro phagocytes	2mM of arginine in culture media.	Increased phagocytic activity.		(J. Alejandro Buentello, Reyes-Becerril, Romero-Geraldo, & Ascencio-Valle, 2007)
Jian carp (*Cyprinus carpio* var. Jian)	In vivo	Fish fed graded levels of dietary arginine. Challenge test: injection of *Aeromonas hydrophila*	Increased serum: C3 and C4 contents, hemaglutination titre. (dietary arginine up to 18.5g/kg diet). Increased serum IgM, lysozyme activity and leukocytes phagocytosis. (dietary arginine up to 16.1g/kg diet).		(G. Chen et al., 2015)

			Up-regulation of mRNA expression of inflammatory cytokines (IL-1b, TNF-α, TGF-β) due to arginine supplementation. Increased survival rates following *Aeromonas hydrophila* infection (dietary arginine containing 16.1-21.9g/kg diet).		
Yellow catfish (*Pelteobagrus fulvidraco*)	In vivo	Fish fed graded levels of dietary arginine. Challenge test: injection of *Aeromonas hydrophila*	Increased arginase, nitric oxide synthase, lysozyme activities, phagocytic index and respiratory burst (diet containing 2.74% arginine).	Survival rate was not affected after bacterial challenge.	(Q. Zhou, Jin, Elmada, Liang, & Mai, 2014)
Blunt snout bream (*Megalobrama amblycephala*)	In vivo	Fish fed graded levels of dietary arginine.	Increased serum and hepatic total nitric oxide synthase. **Reduced** growth in fish fed diets with excessive arginine.	No effect on plasma superoxide dismutase.	(Ren et al., 2013)
Largemouth bass (*Micropterus salmoides*)	In vivo	Fish fed graded levels of dietary arginine.	Increased serum lysozyme activity, serum protein and respiratory burst of head kidney leucocytes.	Complement activity was not affected.	(H. Zhou, Chen, Qiu, Zhao, & Jin, 2012)

Golden pompano (*Trachinotus ovatus*)	In vivo	Fish fed graded levels of dietary arginine. Challenge test: injection of *Vibrio harveyi*	Increased serum and hepatic total nitric oxide synthase and lysozyme activities. Increased survival rates following *Vibrio harveyi* infection (dietary arginine level of 2.65%).		(Lin et al., 2015)
Yellow grouper (*Epinephelus awoara*)	In vivo	Fish fed graded levels of dietary arginine.	Increased serum and hepatic total nitric oxide synthase (as dietary arginine increased).		(Q. C. Zhou, Zeng, Wang, Xie, & Zheng, 2012)
Darkbarbel catfish (*Pelteobagrus vachelli*)	In vivo	Fish fed graded levels of dietary arginine. Challenge test: injection of *Aeromonas hydrophila*	Increased respiratory burst activity of head kidney macrophages (dietary arginine/lysine level of 4.86%/8.27%). Increased serum nitric oxide synthase activity (as dietary arginine increased).	Survival rate was not affected after bacterial challenge	(Feng, Qing-hui, Wei, Kang-sen, & Wen-bing, 2011)

Senegalese sole (*Solea senegalensis*)	In vivo	Fish fed graded levels of dietary arginine. Challenge test: injection of *Photobacterium damselae* subsp. *piscicida* (strain PC566.1)	Increased respiratory burst activity and nitric oxide production of head kidney leucocytes with higher arginine supplementations. Increased lysozyme, alternative complement pathway, and peroxidase activities (dietary arginine level of 5.7 and 6.9 g^{-1} N). Increased HIF-1, HAMP-1, MIP1-alpha and gLYS expression values (dietary arginine level of 5.7 and 6.9 g^{-1} N).		(B. Costas et al., 2011)
Channel catfish (*Ictalurus punctatus*)	In vitro: 1-Primary cell cultures of head-kidney macrophages 2- Naïve peripheral blood lymphocytes	Supplementation of culture media with and/or glutamine.	Increased macrophage phagocytosis and killing ability against *Edwardsiella ictaluri*. (1mM of arginine in culture media) Increased proliferation of naïve T- and B-lymphocytes upon mitogenic exposure (0.5 mM of arginine + glutamine in culture media).		(Pohlenz, Buentello, Mwangi, & Gatlin, 2012)

Yellow catfish (*Pelteobagrus fulvidraco*)	In vivo	Fish fed graded levels of arginine. Challenged to ammonia-nitrogen for 72 h.	Increased anti-ammonia-nitrogen stress ability (dietary arginine level of 2.81%).		(Q. Chen et al., 2016)
Red drum, (*Sciaenops ocellatus*)	In vivo	Fish fed graded levels of dietary arginine and glutamine.	Increased neutrophil oxidative radical production, serum lysozyme, and extracellular and intracellular superoxide anion production of kidney macrophages (dietary level of 1% arginine+1% glutamine).		(Cheng et al., 2011)
Turbot (*Scophthalmus maximus*)	In vivo	Fish fed graded levels of dietary arginine. Challenged to repeated handling, as a chronic stress factor.	Increased monocytes numbers, nitric oxide production, plasma lysozyme, superoxide dismutase and alternative complement pathway activities (in both control and stressed fish, with arginine supplements).		(Benjamín Costas et al., 2012)
Hybrid striped bass (*Morone chrysops×Morone saxatilis*)	In vivo	Fish fed graded levels of dietary arginine.	Increased neutrophil oxidative radical production, serum lysozyme, extracellular and intracellular superoxide anion production of		(Cheng, Gatlin, & Buentello, 2012)

			kidney macrophages (dietary level of 1% arginine).		
Channel catfish (*Ictalurus punctatus*)	In vivo	Fish fed graded levels of dietary arginine and/or glutamine. Challenged against *Edwardsiella ictaluri*.	Increased survival rates following *Edwardsiella ictaluri* infection (dietary arginine level of 2%).		(J. A. Buentello & Gatlin, 2001)
Channel catfish (*Ictalurus punctatus*)	In vivo	Fish fed graded levels of dietary arginine and/or glutamine. Vaccination against *Edwardsiella ictaluri*.	Increased antibody titers in plasma (dietary level of 4% arginine; 2% glutamine and a combination of both). Increased responsiveness of spleen and head-kidney lymphocytes against *E. ictaluri* (dietary level of 4% arginine; 2% glutamine). Increased protein content in head-kidney (dietary level of 4% arginine)		(Pohlenz, Buentello, Criscitiello, et al., 2012)

Turbot (*Scophthalmus maximus L.*)	In vivo	Fish fed graded levels of dietary arginine or/and glutamine. Challenged against *Edwardsiella ictaluri*.	Increased lysozyme and glutathione peroxidase activity in serum; increased inducible nitric oxide synthase activity in serum and liver, and arginase I gene expression in liver (as dietary arginine and glutamine increased). Increased survival rates following *Edwardsiella ictaluri* infection (as dietary arginine increased). Increased respiratory burst activity in head-kidney macrophages (as dietary arginine increased).	No effect on fish growth performance.	(Zhang et al., 2017)
European seabass (*Dicentrarchus labrax*)	In vivo	Fish fed graded levels of dietary arginine. Challenged against *Vibrio anguillarum.*	**Decreased** extracellular superoxide and nitric oxide values. **Decreased** survival rate after bacterial challenge. **Down-regulation** of interleukins and immune-cell marker transcripts (dietary level of 1 and 2% arginine).		(Azeredo et al., 2015)

Citrulline

Citrulline is a non-essential AA, constituent of body proteins. Arginine and citrulline are linked in several metabolic reactions described in Figure 9. As an essential AA, the little contribution for arginine's de novo synthesis in Elasmobranches and ureogenic teleost, is only through the conversion of citrulline via argininosuccinate synthase and lyase in the liver (Fig.9) (Mommsen, Moon, & Plisetskaya, 2001). However, little information is available regarding the efficacy of citrulline to replace arginine in fish nutrition (Li et al., 2009).

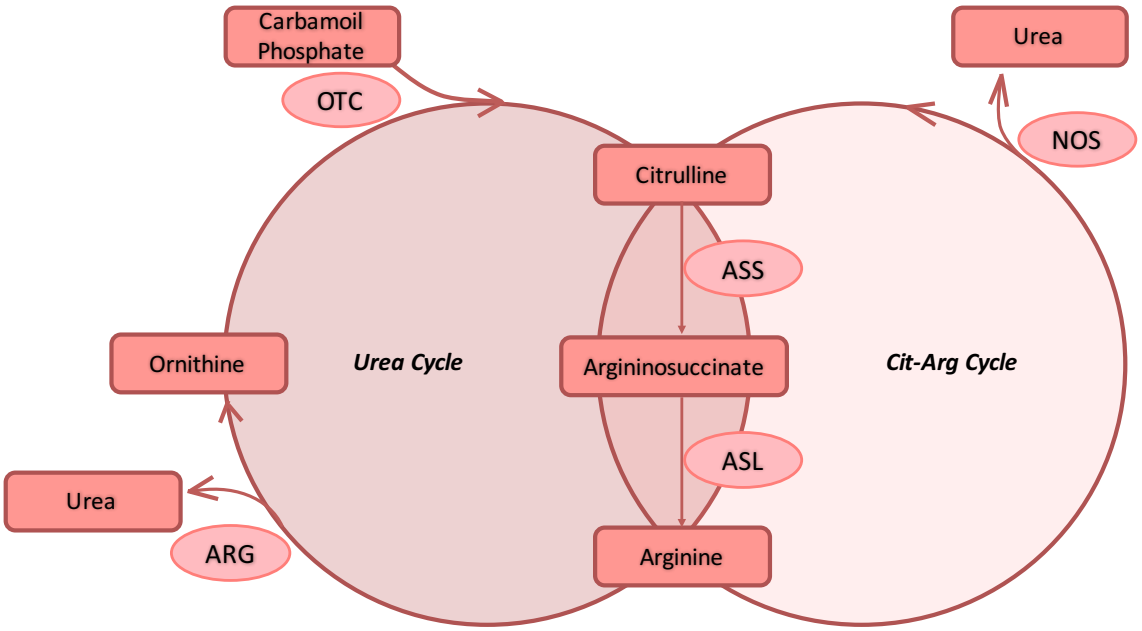


***Figure 9**– Citrulline metabolism. ASS argininosuccinate synthase, ASL argininosuccinate lyase, ARG arginase, OTC ornithine carbamoiltransferase, NOS nitric oxide synthase, Cit-Arg Cycle citrulline arginine cycle.*

In mammals, it has been reported that citrulline might offer a safe alternative to arginine for improving macrophage function under certain metabolic conditions (Breuillard, Bonhomme, Couderc, Cynober, & De Bandt, 2014) Moreover, pre-treatment with citrulline stimulated intestinal production of secretory immunoglobulin A in mice, which is the first line of host defences against environmental pathogens (Batista et al., 2011). Indeed, few studies have approached the effect of citrulline surplus on immune responses in higher

vertebrates, and to the best of our knowledge there currently are not available data in fish.

Objectives

Skin mucosal immunity is a key component of the innate immune and its usage for monitoring fish health status has already been proven. As such, in this study we hypothesize whether SALT immune parameters are affected by nutritional changes and immune challenges. The goal is to evaluate the SALT's capacity to describe fish condition, and its applicability to be used as a biomarker in fish farms.

The other main goal of this thesis is to provide a better understanding of the influence of arginine, citrulline and methionine supplementation on mucosal immune mechanisms and inflammatory response. This knowledge should allow the development of functional commercial diets, hence getting better farming results in terms of growth and disease susceptibility of European seabass.

Material and Methods

Trial 1 - EFFECTS OF METHIONINE AVAILABILITY ON EUROPEAN SEABASS IMMUNE CONDITION AND INFLAMATORY RESPONSE

Rearing conditions

At i3S (Porto, Portugal) fish rearing facilities, European seabass (±8.5g) juveniles were maintained under standard culture conditions for a quarantine period of two weeks. In a recirculation seawater system (Temperature: 20 ± 0.5 ˚C; Salinity: 35 ppt; Photoperiod: 10h dark, 14h light) fish were then distributed into 12 fiberglass tanks (200 l; n=50) for an acclimatization period of 1 week. Temperature was maintained by a water heater/cooler system. Oxygen saturation was held at around 7.3 mg/L and photoperiod automatically controlled. Both nitrite and ammonium levels were daily recorded and its levels controlled by a water ozoniser system. Water renovations and system cleanings were performed twice a week. Dietary treatments were randomly assigned to triplicate tanks and fish were fed three times a day by hand (9.30 am, 1.30 pm and 5.30 pm).

Diets composition

Four diets were formulated and manufactured by Sparos Lda. (Olhão. Portugal). A control diet (CTRL) was formulated to include an indispensable AA profile meeting the ideal pattern estimated for European seabass (Kaushik, 1998). Two other diets, identical to the CTRL were supplemented with DL-Methionine at 0.5% and 1% MET 0.5 and MET 1, respectively at the expenses of wheat gluten. A negative control diet (NCTRL) was also formulated to be deficient in methionine. Main ingredients were ground (below 250 μm) in a micropulverizer hammer mill (SH1; Hosokawa Micron B.V., Doetinchem, The Netherlands). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca S.L., Granollers, Spain). All diets were manufactured by temperature- controlled

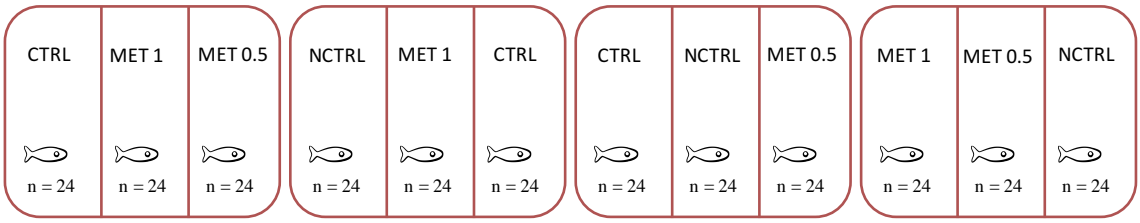
extrusion (pellet sizes: 1.5 mm) by means of a low-shear extruder (P55; Italplast S.r.l., Parma, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientifics, Oldham, UK) for 4h at 45°C. Formulation of experimental diets is presented in Table 2. Four diets were randomly assigned to triplicate tanks and fish were feed 3 times a day. By hand and *ab libitum* the experimental diets assigned.

Table 2 *- Ingredients of the experimental diets.*

Ingredients	CTRL %	NCTRL %	MET 0.5 %	MET 1 %
Fishmeal LT70 (South American)	11.000	5.000	11.000	11.000
Fishmeal 60	17.000	7.500	17.000	17.000
Soy protein concentrate	12.000	18.500	12.000	12.000
Wheat gluten	8.000	10.700	7.700	7.400
Corn gluten	4.000	9.000	4.000	4.000
Soybean meal 48	14.000	14.000	14.000	14.000
Rapeseed meal	6.000	5.300	6.000	6.000
Wheat meal	10.000	10.800	9.800	9.600
Fish oil	8.500	9.000	8.500	8.500
Rapeseed oil	5.000	5.700	5.000	5.000
Vitamin and mineral premix	1.000	1.000	1.000	1.000
Brewer's yeast	3.000	3.000	3.000	3.000
Soy lecithin	0.500	0.500	0.500	0.500
DL-Methionine			0.500	1.000
Total	**100.000**	**100.000**	**100.000**	**100.000**
Pellet size, mm	1.5	1.5	1.5	1.5

Feeding trial

The feeding trial lasted for 4 weeks, in order to assess the effect of short -term dietary supplementation or deficiency of methionine. At the end of each period, twelve fish per tank were sacrificed by an anesthetic overdose with 2-phenoxyethanol and individually weighed (Fig. 10). Skin mucus was gently collected by swabbing from head to tail on both sides of the fish as seen in figure 11, and avoiding contamination from urine, faeces and blood. Mucus samples were frozen and stored at -80ºC for further analysis.



- n = 12 / tank → 2 weeks
- n = 12 / tank→ 4 weeks

***Figure 10**- Representative diagram of the Feeding Trial.*

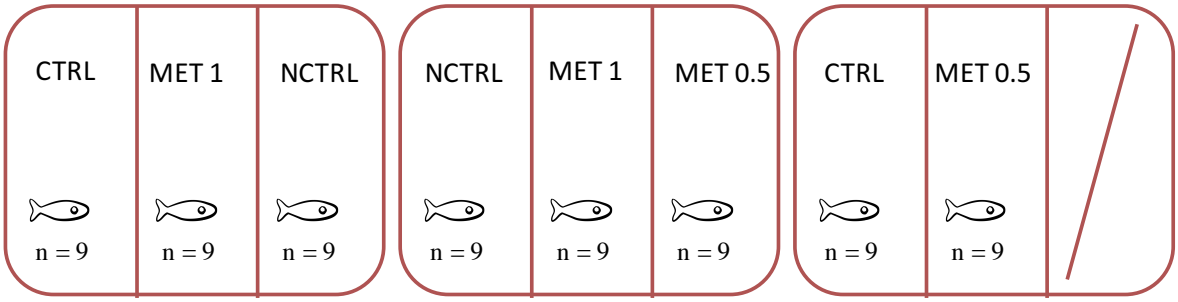
Figure 11- Mucus sampling.

Bacterial growth and inoculum preparation

Phdp, strain PP3, was kindly provided by Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto. Portugal) and isolated from yellowtail (*Seriola quinqueradiata*; Japan) by Dr. Andrew C. Barnes (Marine Laboratory, Aberdeen, UK). Bacteria were routinely cultured at 22°C in tryptic soy broth (TSB) or tryptic soy agar (TSA) (both from Difco Laboratories) supplemented with NaCl to a final concentration of 2% (w/v) (TSB-2 and TSA-2, respectively) and stored at – 80°C in TSB-2 supplemented with 15% (v/v) glycerol. To prepare the inoculum for injection into the fish peritoneal cavities, 100 μL of stocked bacteria were cultured overnight at 22°C on TSA-2. Exponentially growing bacteria were collected from the TSA-2 and re-suspended in sterile TSB-2. According to the pre-challenge previously performed, the intended bacterial concentration to kill 50% of the fish (LD50) was obtained by absorbance reading and adjustment against its growth curve to 5 x 10^4 colony forming units (cfu) ml^{-1}. Bacteria concentration was confirmed by plating the resulting cultures on TSA-2 plates and counting of the colony forming units (cfu) ml^{-1}.

Time-course trial

Immediately after the 4 weeks sampling, the 26-remaining fish were intraperitoneally (i.p.) injected with 100 μl *Phdp* (5 x 10^4 cfu) (Fig. 13). After i.p. injection, 6 fish from each tank were relocated in a comparable recirculation system (Temperature: 24 ± 0.5 °C; Salinity: 35 ppt; Photoperiod: 10h dark: 14h light) and divided in two tanks according to dietary treatment (Fig.12). This experiment was designed to investigate the immunomodulatory effect of methionine during the acute inflammatory response against *Phdp* in fish previously fed the experimental diets. For that purpose, fish were sampled at 4, 24h after challenge. At each sampling time, 3 fish per tank were sacrificed by anesthetic overdose with 2-phenoxyethanol and skin mucus collected.



- n = 3 / tank → 4h
- n = 3 / tank→ 24h
- n = 3 / tank→ 48h

***Figure 12**- Representative diagram of the experimental setup for the time-course trial.*

***Figure 13**- Intraperitoneal injection with Phdp.*

Trial 2- EFFECTS OF ARGININE AND CITRULLINE AVAILABILITY ON EUROPEAN SEABASS IMMUNE CONDITION

Rearing conditions

At i3S fish rearing facilities, European seabass (8.4 g ± 0.39 g) were maintained under standard culture conditions for a quarantine period of one weeks. In a recirculation seawater system (Temperature: 20 ± 0.5 ˚C; Salinity: 35 ppt; Photoperiod: 10h dark: 14h light) fish were then distributed into 12 fiberglass tanks (200 l; n=50) for an acclimatization period of 1 week. Temperature was maintained by a water heater/cooler system. Oxygen saturation was maintained at around 7.3 mg/L and photoperiod automatically controlled. Both nitrite and ammonium levels were daily recorded and its levels controlled by a water ozoniser system. Water renovations and system cleanings were performed twice a week. Dietary treatments were randomly assigned to triplicate tanks and fish were fed three times a day by hand (9.30 am. 1.30 pm and 5.30 pm) until apparent satiety.

Diets composition

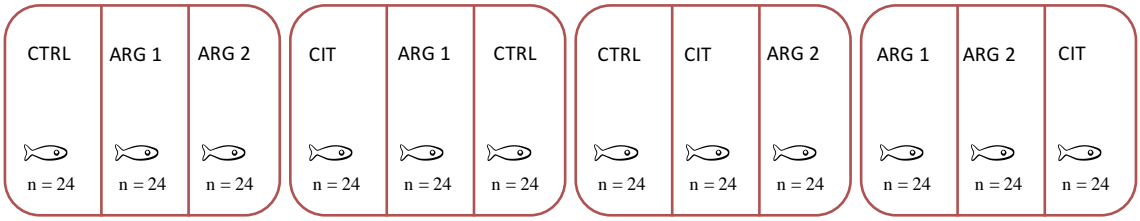
Four diets were formulated and manufactured by Sparos Lda, (Olhão. Portugal). A control diet (CTRL) was formulated to include an indispensable AA profile meeting the ideal pattern estimated for European seabass (Kaushik, 1998). Two other diets, identical to the CTRL were supplemented with DL-Arginine and DL-citrulline at 0.5 % dry matter (ARG1 and CIT1, respectively) at the expenses of wheat meal. A third diet was formulated by supplementing DL-arginine at 1 % dry matter (ARG2). Main ingredients were ground (below 250 μm) in a micropulverizer hammer mill (SH1; Hosokawa Micron, B.V., Doetinchem, The Netherlands). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca, S.L., Granollers, Spain). All diets were manufactured by temperature-controlled extrusion (pellet sizes: 1.5 mm) by means of a low-shear extruder (P55; Italplast, S.r.l., Parma, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientifics, Oldham, UK) for 4h at 45 ˚C. Formulation of experimental diets is presented in Table 3.

Table 3 - Ingredients of the experimental diets.

Ingredients	CTRL	ARG1	ARG2	CIT
	%	%	%	%
Fishmeal LT70 (South American)	5.000	5.000	5.000	5.000
Porcine blood meal	2.000	2.000	2.000	2.000
Poultry meal 65	5.000	5.000	5.000	5.000
Potato concentrate	10.000	10.000	10.000	10.000
Wheat glúten 1	13.500	13.500	13.500	13.500
Corn glúten 2	30.000	30.000	30.000	30.000
Soybean meal 48	5.000	5.000	5.000	5.000
Wheat meal	8.000	7.500	7.000	7.500
Fish oil 3	10.000	10.000	10.000	10.000
Rapeseed oil 4	5.500	5.500	5.500	5.500
Vitamin and mineral premix 5	1.000	1.000	1.000	1.000
Binder (natural zeolite)	1.000	1.000	1.000	1.000
Antioxidant 6	0.200	0.200	0.200	0.200
Sodium propionate	0.100	0.100	0.100	0.100
Monocalcium phosphate 7	2.000	2.000	2.000	2.000
L-Arginine		0.500	1.000	
L-Citrulline				0.500
L-Histidine	0.300	0.300	0.300	0.300
L-Lysine	0.800	0.800	0.800	0.800
L-Threonine	0.200	0.200	0.200	0.200
DL-Methionine	0.400	0.400	0.400	0.400
Total	100.000	100.000	100.000	100.000
Pellet size. mm	1.5	1.5	1.5	1.5

Feeding trial

The feeding trial lasted for 4 weeks in order to assess the effect of short -term AA dietary supplementation. After 2 and 4 weeks of feeding, twelve fish per tank were sacrificed by anesthetic overdose with 2-phenoxyethanol and individually weighed (Figure 14). Skin mucus was gently collected by swabbing from gill to tail on both sides of the fish, and avoiding contamination from urine, feces and blood. Mucus samples were frozen and stored at -80ºC for further analysis.



- n = 12 / tank → 2 weeks
- n = 12 / tank→ 4 weeks

***Figure 14**- Representative diagram of the Feeding Trial.*

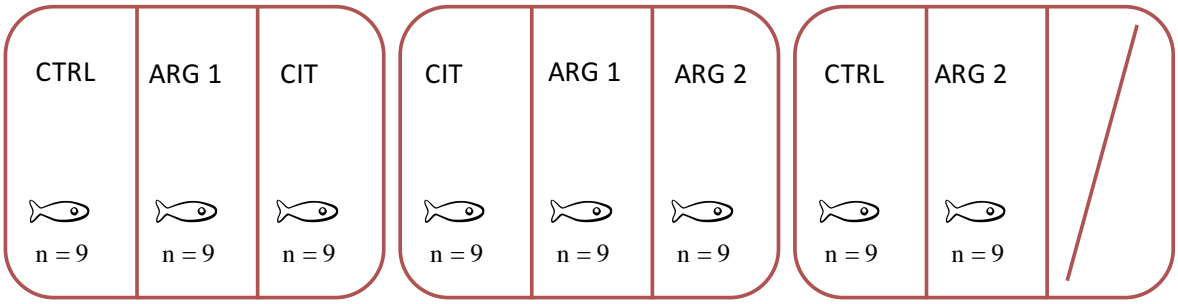
Bacterial growth and inoculum

Bacteria were grown and harvested as described above. For this trial, the intended bacterial concentration to kill 50 % of the fish (LD_{50}) was obtained: 5 x 10^3 colony forming units (CFU) ml^{-1}. Bacteria concentration was confirmed by plating the resulting cultures on TSA-2 plates and counting of the CFU ml^{-1}.

Time-course trial

Immediately after the 4 weeks sampling, the 26 remaining fish were intraperitoneally injected with 100 µl *Phdp* (5 x 10^3 cfu). After i.p. injection, 6 fish from each tank were relocated in a comparable recirculation system (Temperature: 24 ± 0.5 ˚C; Salinity: 35 ppt; Photoperiod: 10h dark: 14h light) and divided in two tanks according to dietary treatment (Fig.15). This experiment was designed to investigate the immunomodulatory effect of methionine during the acute

inflammatory response against *Phdp* in fish previously fed the experimental diets. For that purpose, fish were sampled at 4. 24 and 48h after challenge. At each sampling time. 3 fish per tank were sacrificed by anesthetic overdose with 2-phenoxyethanol and skin mucus collected.



- n = 3 / tank → 4h
- n = 3 / tank→ 24h
- n = 3 / tank→ 48h

***Figure 15**- Representative diagram of the experimental setup for the time-course trial.*

Humoral parameters analytical procedures

Bactericidal Activity

Phdp strain PP3 was used to determine the bactericidal activity of the mucus sample. After being cultured for 48 h at 25 ˚C on tryptic soy agar (TSA; Difco Laboratories), the bacteria were inoculated into tryptic soy broth (TSB; Difco Laboratories), both supplemented with NaCl to a final concentration of 1% (w/v) (TSA-1 and TSB-1, respectively). Bacteria in TSB-1 medium were then cultured at the same temperature for 24h, with continuous shaking (100 rpm). Exponentially growing bacteria were collected by centrifugation at 3500 × g for 30 min, re-suspended in sterile HBSS and adjusted to 1×10^6 cfu ml^{-1}. To confirm bacterial concentration of the inoculum, plating serial dilutions of the suspensions were performed onto TSA-1 plates and the number of cfu was counted following incubation at 25 ˚C.

Mucus bactericidal activity was determined following the method of (Stevens Kehrli & Canning 1991) with modifications. Briefly, 20 µl of mucus and 20 µl of *Phdp* (1 ×

10^6 cfu ml^{-1}) were added to triplicate wells of a round-bottom 96-well plate, following incubation 2.5h at 25˚C. Hank´s balanced salt solution (HBSS) was added to some wells instead of mucus as a positive control. To each well. 25 µl of 3-(4.5 dimethyl-2-yl)-2.5-diphenyl tetrazolium bromide (1 mg ml^{-1}; Sigma) were added and incubated for 10 min at 25 ºC to allow the formation of formazan. Plates were then centrifuged at 2000 × g for 10 min and the precipitate was dissolved in 200 µl of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan was measured at 560 nm. Bactericidal activity is expressed as percentage, calculated from the difference between bacteria surviving compared to the number of bacteria from positive controls (100%).

$$\% \textbf{ non viable bacteria} = \frac{\textbf{Sample Abs. x 100}}{\textbf{Abs. of the reference sample}}$$

$$\%\textbf{viable bacteria} = \textbf{100} - \% \textbf{ non viable bacteria}$$

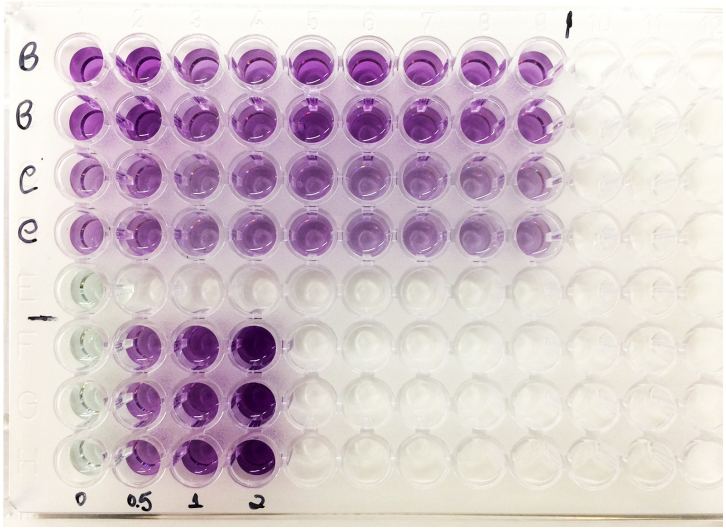


Figure 16- 96-well microplates used in Bactericidal activity test.

Lysozyme

Lysozyme activity was measured using a turbidimetric assay as described by (B. Costas et al., 2011). Briefly. a solution of *Micrococcus lysodeikticus* (0.5 mg ml^{-1}.

0.05 M sodium phosphate buffer. pH 6.2) was prepared. In a round-bottom 96-well plate, 15 µl of mucus and 250 µl of the above suspension were added to give a final volume of 265 µl. The absorbance (450 nm) was measured after 0.5 and 10 min in a Synergy HT microplate reader, Biotek, at 25ºC. A standard curve was developed by using lyophilized hen egg white lysozyme (Sigma), which was serially diluted in sodium phosphate buffer (0.05 M. pH 6.2). Using the formula of the standard curve the amount of lysozyme in the sample was calculated. All analysis were conducted in triplicates.

Alternative Complement Pathway

Alternative complement pathway (ACP) activity was estimated as described by (Sunyer & Tort, 1995). The following buffers were used: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, as previous one but containing 20 mM EDTA; and Mg- EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA. Horse red blood cells (RaRBC; Probiologica Lda, Portugal) were used for ACP determination. RaRBC were washed four times in GVB and re-suspended in GVB to a concentration of 2.5×10^{8} cells ml^{-1}. Ten µl of RaRBC suspension were then added to 40 µl of serially diluted mucus in Mg-EGTA-GVB buffer. Samples were incubated at room temperature for 100 min with regular shaking. The reaction was stopped by adding 150 µl of cold EDTA-GVB. Samples were then centrifuged and the extent of haemolysis was estimated by measuring the optical density of the supernatant at 414 nm in a Synergy HT microplate reader Biotek. The ACH50 units were defined as the concentration of mucus giving 50% haemolysis of RaRBC. All analysis were conducted by triplicates.

Peroxidase Activity

Total peroxidase activity in mucus was measured following the procedure described by (Quade & Roth, 1997). Briefly, 15 µl of mucus were diluted with 135 µl of HBSS without Ca^{+2} and Mg^{+2} in flat- bottomed 96-well plates. Then, 50 µl of 20 mM 3.3',5.5'-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 µl of 5 mM H_2O_2 were added. The color-change reaction was stopped after 2 min by adding

50 µl of 2 M H_2SO_4 and the optical density was read at 450 nm in a Synergy HT microplate reader, Biotek. The wells without mucus were used as blanks. The peroxidase activity (units ml^{-1} mucus) was determined defining one unit of peroxidase as that which produces an absorbance change of 1 OD. All analysis were conducted in triplicates.

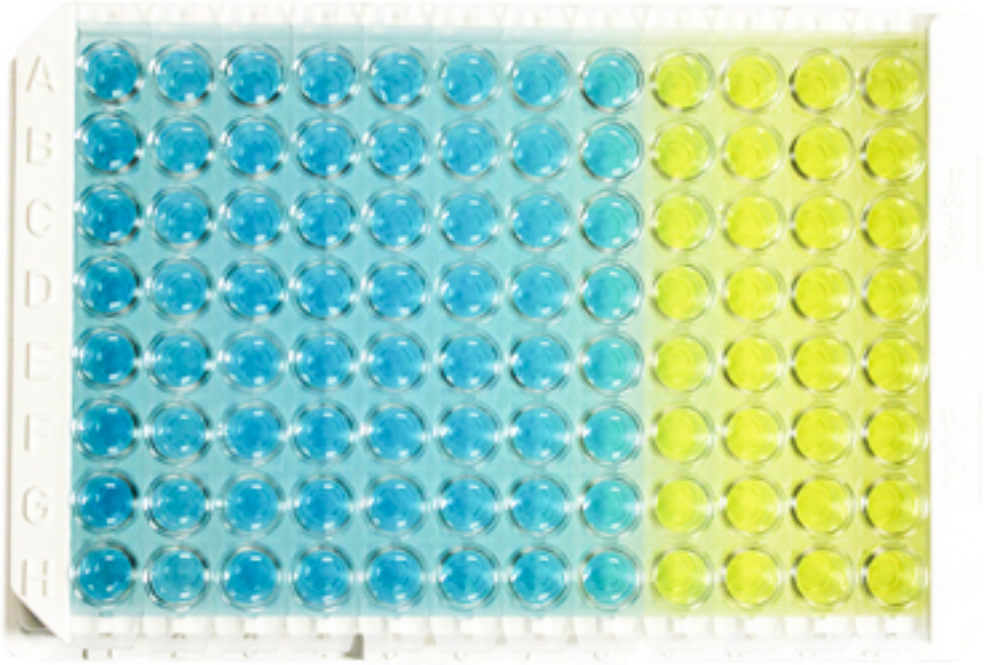


Figure 17- 96-well microplates used in Bactericidal activity test.

Anti-Protease Activity

The method described by (Ellis, 1990) was modified and adapted for 96-well microplates. Briefly, 10 µl of mucus were incubated with the same volume of a trypsin solution (5 mg ml^{-1} in $NaHCO_3$, 5 mg ml^{-1}, pH 8.3) for 10 min at 22°C in polystyrene microtubes. To the incubation mixture, 100 µl of phosphate buffer (NaH_2PO_4, 13.9 mg ml^{-1}, pH 7.0) and 125 µl of azocasein (20mg ml^{-1} in $NaHCO_3$, 5mg ml^{-1}, pH 8.3) were added and incubated for 1h at 22°C. Finally, 250 µl of trichloroacetic acid were added to each microtube and incubated for 30 min at 22oC. The mixture was centrifuged at 10 000 x g for 5 min at room temperature.

Afterwards, 100 µl of the supernatant was transferred to a 96 well-plate that previously contained 100 µl of NaOH (40 mg ml^{-1}) per well. The OD was read at 450nm. Phosphate buffer in place of mucus and trypsin served as blank whereas the reference sample was phosphate buffer in place of mucus. The percentage of trypsin activity inhibition was calculated as follows:

$$\%\ \textbf{non inhibited trypsin} = \frac{\textbf{Sample Abs. x 100}}{\textbf{Abs. of the reference sample}}$$

$$\%\ \textbf{inhibited trypsin} = \textbf{100} - \%\ \textbf{non inhibited trypsin}$$

Data Analysis

All results are expressed as mean ± standard deviation (mean ± SD). All variables were checked for normality and homogeneity of variance, by using the Shapiro-Wilk and the Levene test, respectively. Data was analyzed by two-way ANOVA, with time and diet as factors and followed by Tukey post-hoc test to identify differences in the experimental treatments. Variable transformation was applied when homogeneity and normality were not achieved. A nonparametric test (Kruskal-Wallis H-test) was performed, if the above assumptions where still not achieved. And a pairwise comparison to obtain the revealed differences.
All statistical analyses were performed using the computer package SPSS for MAC. The level of significance used was $P \leq 0.05$ for al statistical tests.

RESULTS

Trial 1 - EFFECTS OF METHIONINE AVAILABILITY ON EUROPEAN SEABASS IMMUNE CONDITION

FEEDING TRIAL

In methionine feeding trail, no dietary effects were observed on mucus bactericidal and peroxidase activities (Figs. 18 and 21), whereas peroxidase values augmented in time regardless of dietary treatment. Furthermore, a slight but significant increase of ACP levels was observed in fish fed CTRL diet at the end of the feeding trail compared to those sampled at 2 weeks (Fig. 19). Seabass fed the Met1 diet presented a decrease in lysozyme levels compared to those fed the CTRL diet at 2 weeks, while after 4 weeks of feeding trail, lysozyme activity decreased significantly in fish fed diet deficient in methionine (NCRT) compared to those fed CTRL, MET05 and MET1 diets (Fig.20). Moreover, those levels were observed to decrease between 2 and 4 weeks, within the group fed NCRT diet. Finally, there was no anti-trypsin activity detectable in skin mucus in all experimental diets.

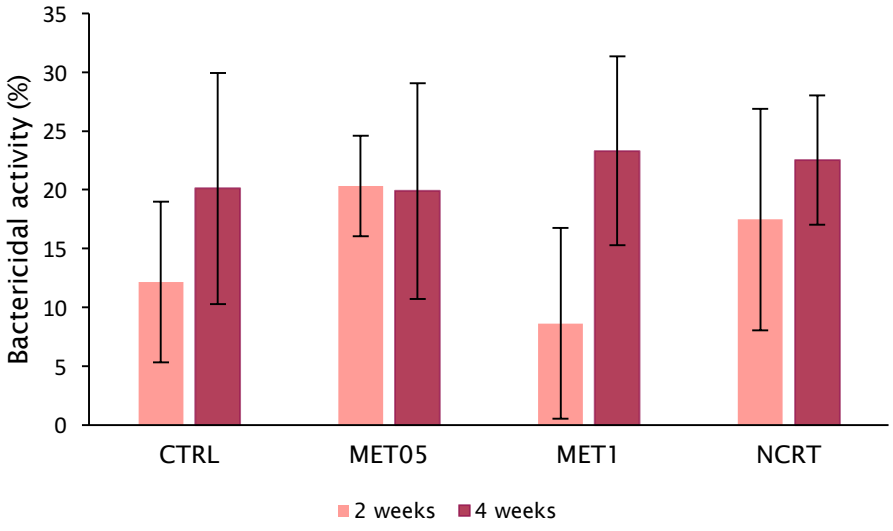


***Figure 18**- Bactericidal activity in mucus of European sea bass fed different diets for 2 (salmon columns) and 4 (bordeaux columns) weeks. Data are expressed as means ± standard error of the mean (SEM) (n = 12). Vales were cos-transformed before being treated statistically (one-way ANOVA; P < 0.05).*

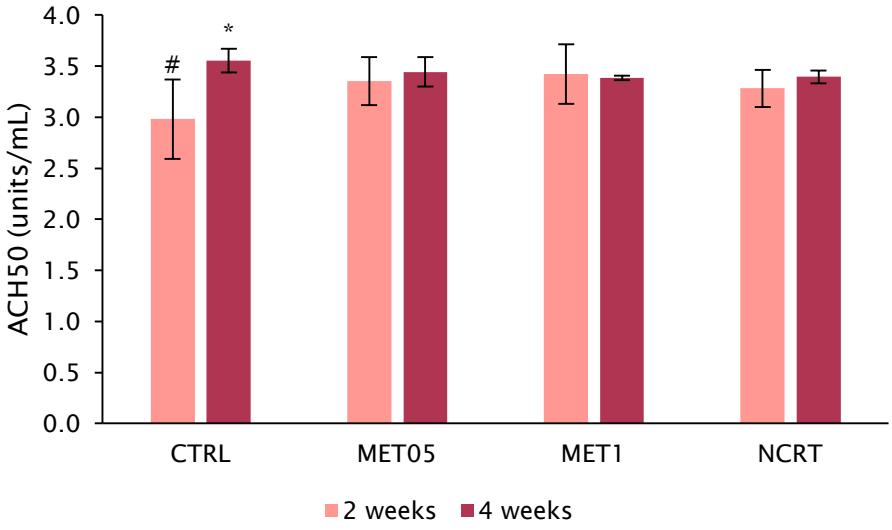


Figure 19 *- Alternative complement pathway in mucus of European sea bass fed different diets for 2 (salmon columns) and 4 (bordeaux columns) weeks. Data are expressed as means ± SEM (n = 12), and analyzed using the Kruskal–Wallis test (P < 0.05).*

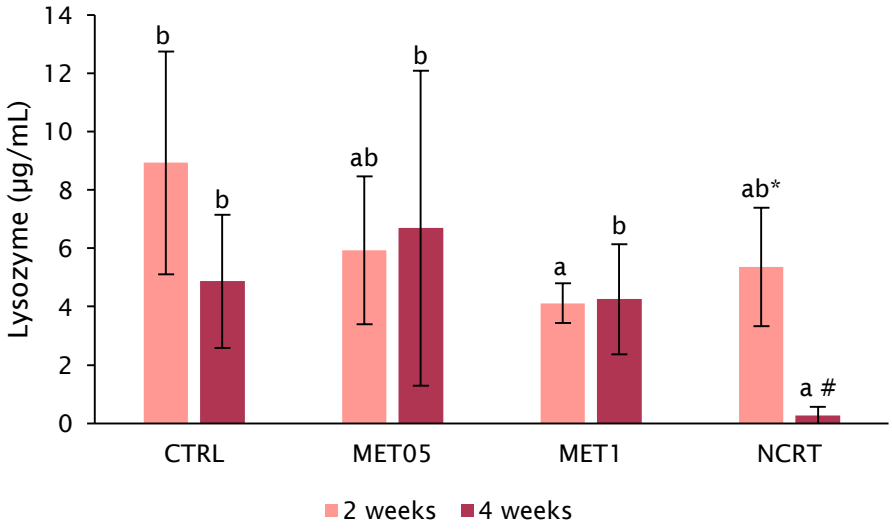


Figure 20*- Lysozyme in mucus of European sea bass fed different diets for 2 (salmon columns) and 4 (bordeaux columns) weeks. Data are expressed as means ± SEM (n = 12), and analyzed using the Kruskal–Wallis test (P < 0.05). Different letters stand for significant differences within dietary treatments, while different symbols indicate significant differences attributed to time.*

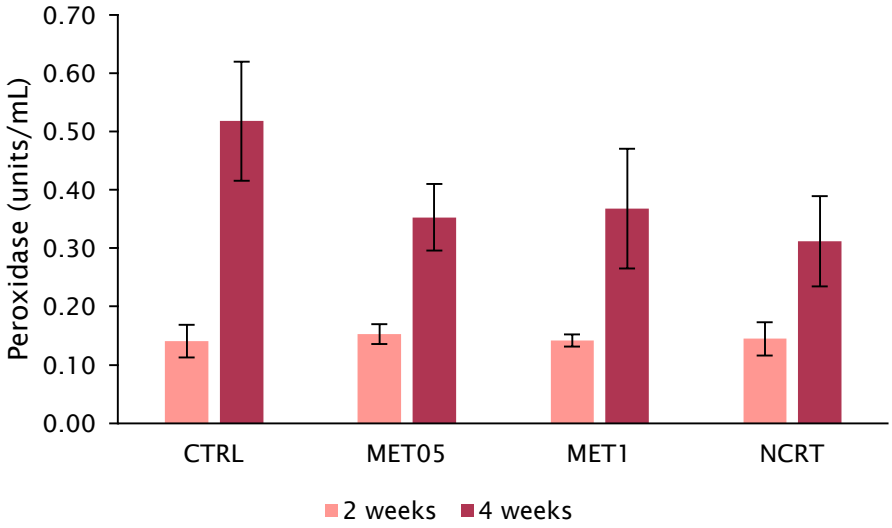


***Figure 21**- Peroxidase in mucus of European sea bass fed different diets for 2 (salmon columns) and 4 (bordeaux columns) weeks. Data are expressed as means ± SEM (n = 12), and analysed using the Kruskal–Wallis test (P < 0.05).*

TIME-COURSE TRIAL

Dietary methionine resulted in significant effects on lysozyme and peroxidase levels after a bacterial infection, but not in ACH50 activity (Fig.22). Fish fed diet MET0.5 presented a significant decrease in skin mucus lysozyme values compared to fish fed the CTRL diet (Fig.23). Regarding peroxidase activity, fish fed methionine deficient diet showed higher values than specimens fed MET1 diet (Fig.24).

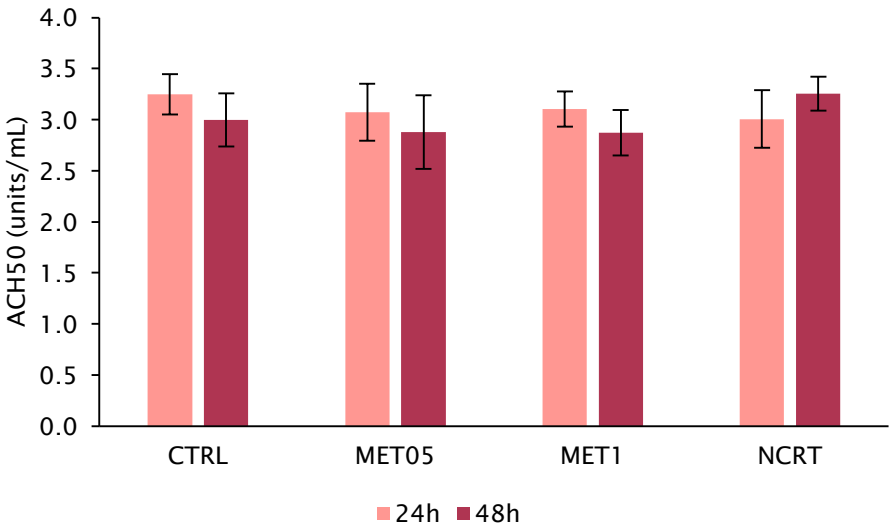


***Figure 22**- Alternative complement pathway in mucus of European sea bass fed dietary treatments at 24 (salmon columns) and 48 hours (bordeaux columns) after peritoneal inflammation. Data are expressed as means ± SEM (n = 9), and analyzed using the one-way ANOVA; (P < 0.05).*

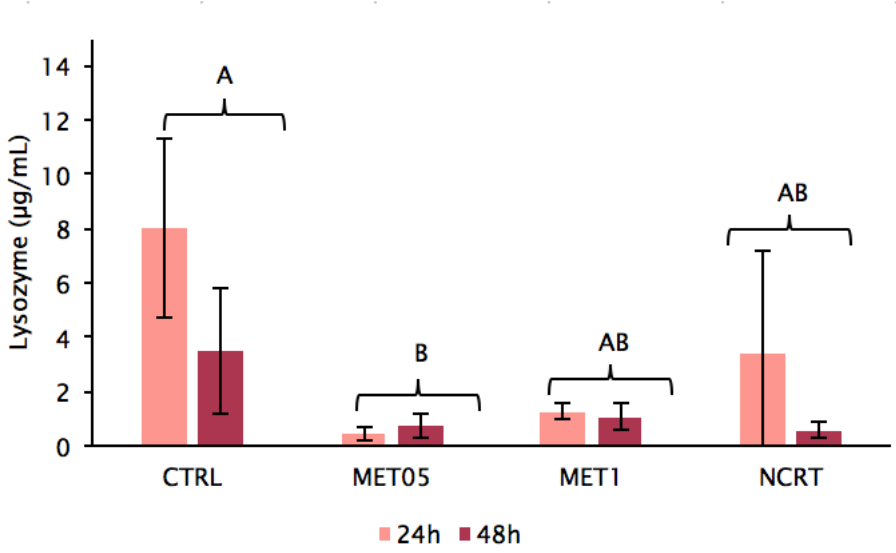


***Figure 23**- Lysozyme in mucus of European sea bass fed dietary treatments at 24 (salmon columns) and 48 hours (bordeaux columns) after peritoneal inflammation. Data are expressed as means ± SEM (n = 9). Vales were arsin-transformed before being treated statistically (one-way ANOVA; P < 0.05).Different capital letters indicate differences among diets regardless time.*

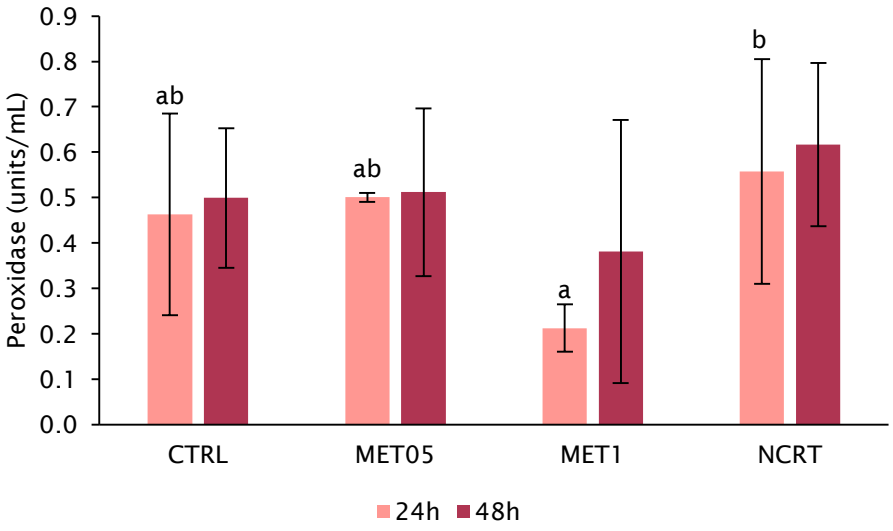


***Figure 24**- Peroxidase in mucus of European sea bass fed dietary treatments at 24 (salmon columns) and 48 hours (bordeaux columns) after peritoneal inflammation. Data are expressed as means ± SEM (n = 9), and analyzed using the Kruskal–Wallis test (P < 0.05). Different letters stand for significant differences within dietary treatments.*

Trial 2- EFFECTS OF ARGININE AND CITRULLINE AVAILABILITY ON EUROPEAN SEABASS IMMUNE CONDITION

FEEDING TRIAL

In arginine feeding trial, no significant changes related to time nor diet were observed for bactericidal activity in none of the 4 feeding groups (Fig.25). In contrast, fish fed the CTRL diet showed an increase in ACH50 and lysozyme activities from 2 to 4 weeks of the feeding period (Figs. 26 and 27, respectively). Peroxidase levels were enhanced between the 2 a 4 week in fish fed the CIT1 diet, whereas these levels decreased over the time in the CTRL group (Fig.28).

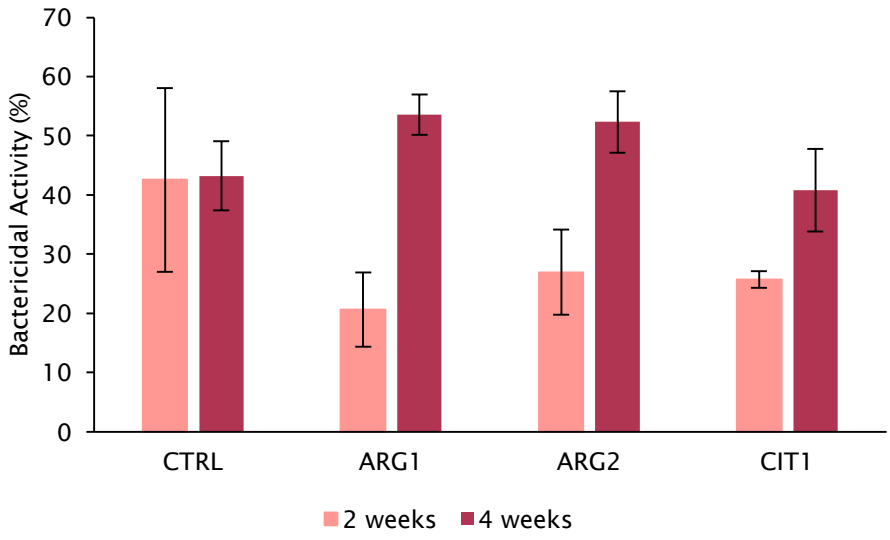


Figure 25 *- Bactericidal activity in mucus of European sea bass fed different diets for 2 (salmon columns) and 4 (bordeaux columns) weeks. Data are expressed as means ± standard error of the mean (SEM) (n = 12). Vales were cos-transformed before being treated statistically (one-way ANOVA; P < 0.05).*

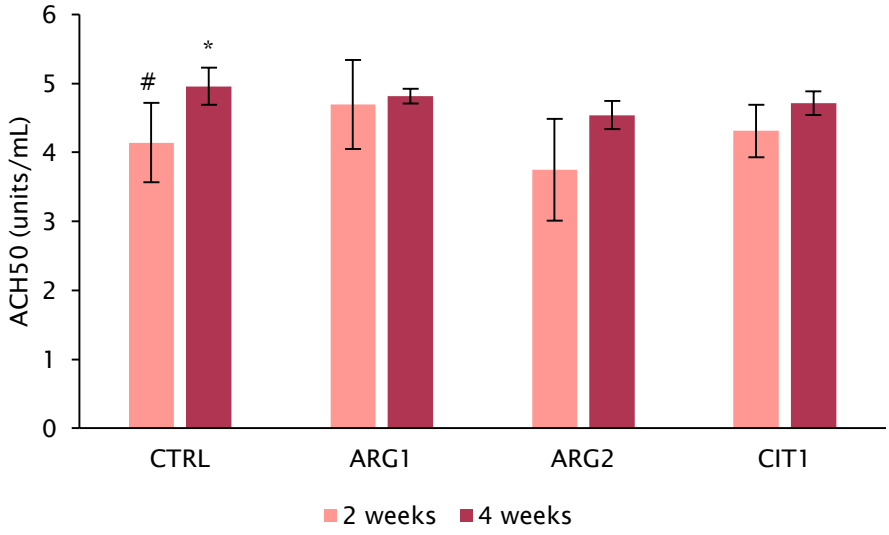


Figure 26 *- Alternative complement pathway in mucus of European sea bass fed different diets for 2 (salmon columns) and 4 (bordeaux columns) weeks. Data are expressed as means ± SEM (n = 12), and analyzed using the Kruskal–Wallis test (P < 0.05). Different symbols indicate significant differences attributed to time.*

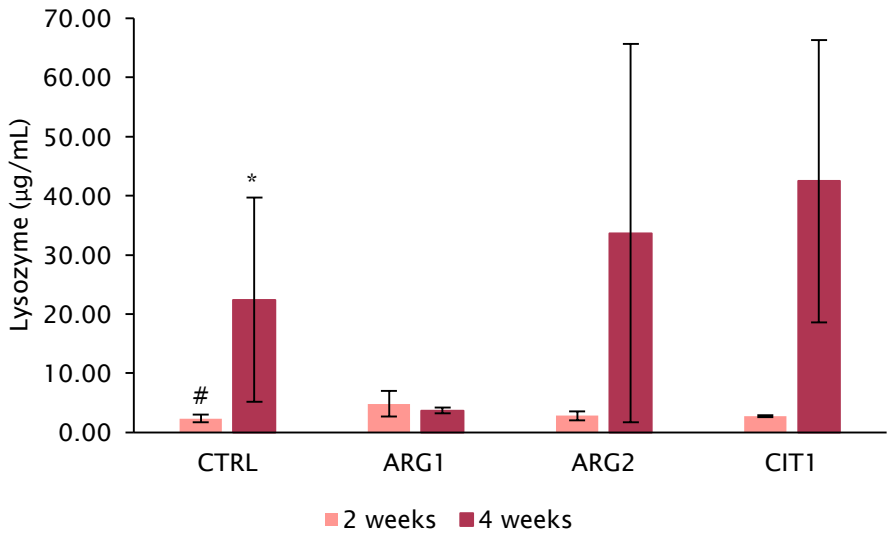


***Figure 27** - Lysozyme in mucus of European sea bass fed different diets for 2 (salmon columns) and 4 (bordeaux columns) weeks. Data are expressed as means ± SEM (n = 12), and analyzed using the Kruskal–Wallis test (P < 0.05). Different symbols indicate significant differences attributed to time.*

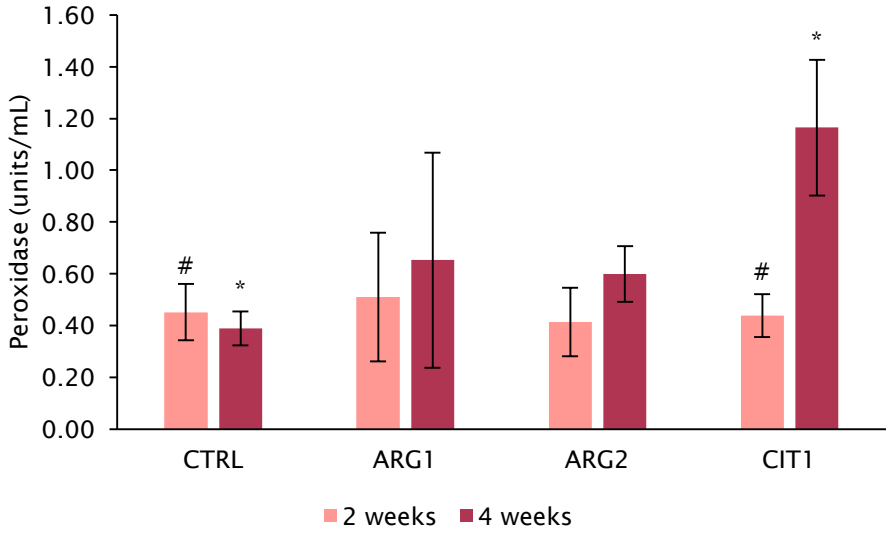


***Figure 28** - Peroxidase in mucus of European sea bass fed different diets for 2 (salmon columns) and 4 (bordeaux columns) weeks. Data are expressed as means ± SEM (n = 12), and analyzed using the Kruskal–Wallis test (P < 0.05). Different symbols indicate significant differences attributed to time.*

TIME-COURSE TRIAL

There were no differences in both Bactericidal and ACH50 activities among all dietary groups (Figs. 29 and 30, respectively). Peroxidase levels significantly decreased over the time following pathogen injection regardless dietary treatments (Fig.32). A general decrease in skin mucus lysozyme levels from 4 to 24 h was observed in all dietary groups, followed by an increase at 48 h in fish fed arginine and citruline supplemented diets compared to the low levels presented by fish fed the CTRL diet. Within ARG1 dietary treatment fish presented a reduction of lysozyme levels from 4 to 24 hours and then at 48 hours they recovered from the depressed levels and exceed the 4 hours' lysozyme values (Fig.31). Moreover, lysozyme activity diminished in fish fed ARG1 and CIT1 compared to fish fed CTRL at 24 h following pathogen injection , although higher levels were observed in fish fed ARG1 compared at 48h.

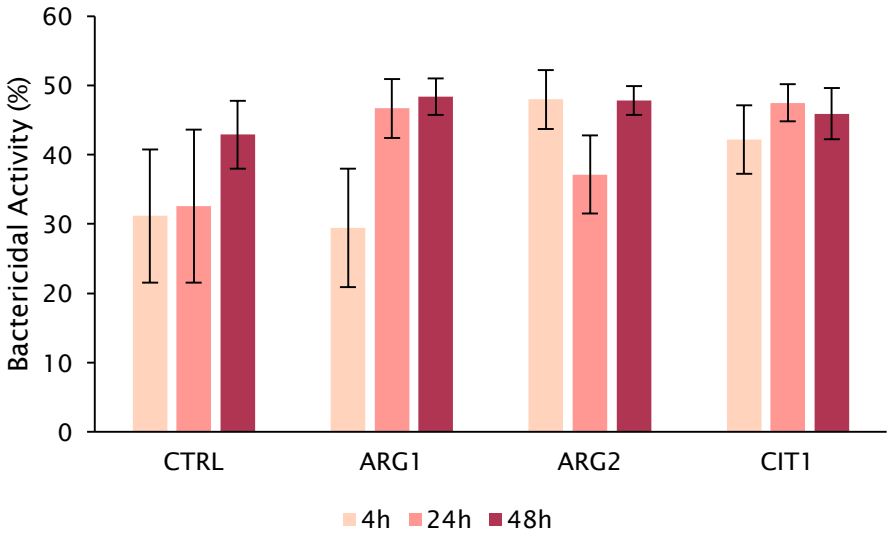


Figure 29 *- Bactericidal activity in mucus of European sea bass fed different diets at 4 (beige columns), 24 (salmon columns) and 48 hours (bordeaux columns) after peritoneal inflammation. Data are expressed as means ± SEM (n = 9), and cos-transformed before being treated statistically (one-way ANOVA; P < 0.05).*

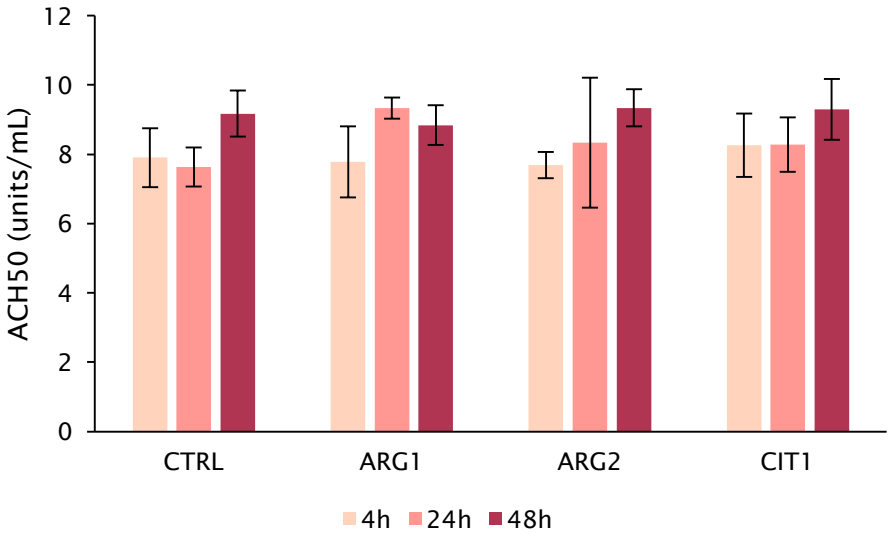


Figure 30 *- Alternative complement pathway in mucus of European sea bass fed different diets at 4 (beige columns), 24 (salmon columns) and 48 hours (bordeaux columns) after peritoneal inflammation. Data are expressed as means ± SEM (n = 9), and analyzed using the Kruskal–Wallis test (P < 0.05).*

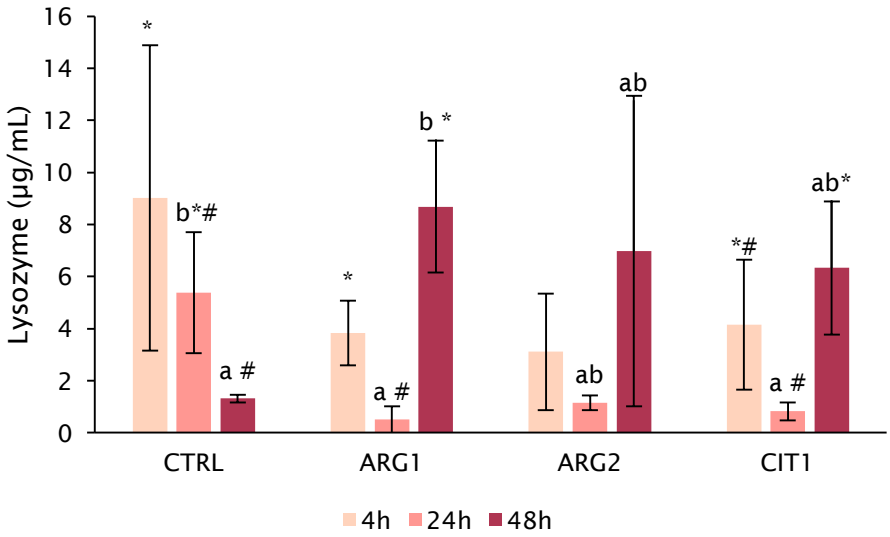


Figure 31 *- Lysozyme in mucus of European sea bass fed different diets at 4 (beige columns), 24 (salmon columns) and 48 hours (bordeaux columns) after peritoneal inflammation. Data are expressed as means ± SEM (n = 9), and analyzed using the Kruskal–Wallis test (P < 0.05). Different letters stand for significant differences within dietary treatments, while different symbols indicate significant differences attributed to time.*

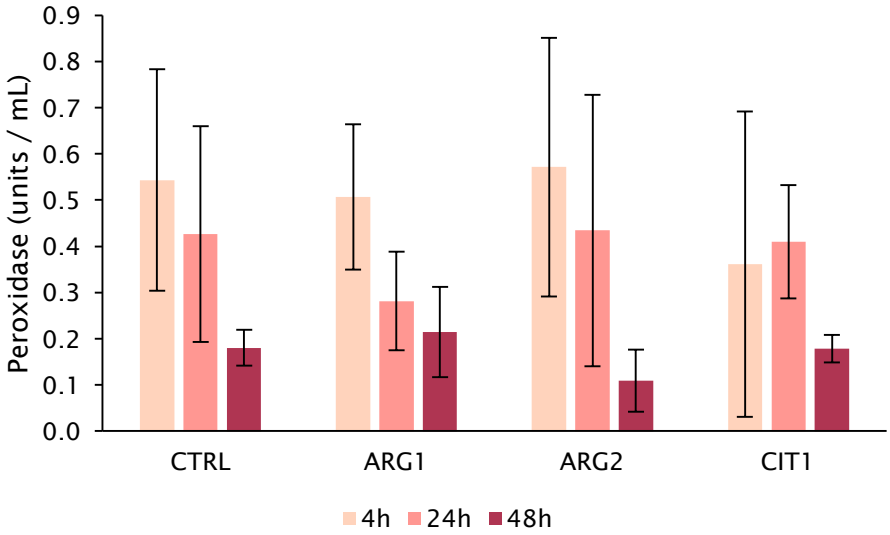


Figure 32 *- Peroxidase in mucus of European sea bass fed different diets at 4 (beige columns), 24 (salmon columns) and 48 hours (bordeaux columns) after peritoneal inflammation. Data are expressed as means ± SEM (n = 9), and Log-transformed before being treated statistically (one-way ANOVA; P < 0.05).*

DISCUSSION

Trial 1 - EFFECTS OF METHIONINE AVAILABILITY ON EUROPEAN SEABASS IMMUNE CONDITION

Over the last years, there was a growing interest in the inclusion of AA in fish functional feeds, aiming an increase of fish health status, which in turn may allow a higher disease resistance. (Andersen, Waagbø, & Espe, 2015). Apart from being an indispensable AA, crucial for fish as energy substrate and protein synthesis, methionine participates in several metabolic pathways with an immune modulatory action. Moreover, this AA is available as feed grade, thus its inclusion in aquafeeds may be obtained at reduced costs (Machado et al., 2015). Taking into account this considerations, several studies were performed, proving methionine's immune status enhancing effect in mammals (Rubin et al., 2007) as well as in fish (Tang et al., 2009). In the present work, fish were fed diets supplemented with methionine levels above the requirement for normal growth for European seabass during 2 and 4 weeks, and also following stimulation with bacteria. To the best of our knowledge, no published work showing the modulatory effects of methionine supplementation on the skin mucus immunity in European seabass is available in the literature.

Regarding the feeding trial, in the absence of infection, dietary methionine appears to have no significant effects on the bactericidal, alternative complement pathway and peroxidase activities. Although not statistically significant, bactericidal activity presented a generally trend to increase over time, so did peroxidase activity, which was significantly higher at the end the feeding trial, regardless of the diet. The enhanced immune effect along time is possibly linked to the fish development and habituation process to the system. Still, the lack of differences among the innate immune parameters measured, between dietary treatments, seems to be linked to effects on skin mucus, since published literature points to a beneficial effect of methionine supplementation during a systemic immune response (Kuang et al., 2012). A possible reason to justify the lack of changes in lysozyme and peroxidase levels may be the absence of an immunological challenge. The presence of a pathogen induces the immune response involving the activation of effector cells such as neutrophils, leading to its consequently degranulation and release of

peroxidase in the local. In fact, lysozyme levels are known to increase in fish subjected to an inflammatory agent (Ellis, 2001).

After 4 weeks of feeding trial fish fed diet deficient in methionine, presented a dramatically drop on the mucosal lysozyme levels. The absence of methionine in fish nutrition compromised the lysozyme's production, even without an immune stimulation. This observation shows the role of methionine as an essential and a limiting AA in fish.

At the end of the 4 weeks of the feeding period, live bacteria were injected in the peritoneal cavity, and dietary modulation in response to an inflammatory stimulus was studied. Methionine is the most common methyl donor in the body, making it essential for cell division events and polyamines synthesis (Grimble, Poshoi, al., & Lauterberg, 1996). However, the current study showed a general inhibitory effect of methionine in mucosal lysozyme and peroxidase values in the time-course trial. At first sight, this may seem contradictory to several other studies, where methionine supplementation followed by bacterial insult, resulted in increased numbers of white blood cells, triggered by the cell proliferation effect of this AA (Kuang et al., 2012). Increased plasma leucocytes numbers, fueled by methionine surplus, should be translated in increased plasma peroxidase and lysozyme activity upon an infection. In fact, Costas et al. 2013 showed that increased lysozyme and peroxidase levels is directly related with neutrophil numbers in Senegalese sole, since these enzymes are generated by neutrophils in response to an inflammatory stimuli (Murray & Fletcher, 1976). In contrast to what has been published for plasma innate parameters, in this work we observed minimum mucus lysozyme activity in methionine fed groups, also, peroxidase values were the lowest in fish fed MET1 diet. The opposite results observed in mucus, may reflect the initial course of an immune response, where leucocytes are recruited to the inflammatory focus from the peripheral tissues (Kuang et al., 2012), such as skin. However, to prove this hypothesis, plasma and peritoneal exudate samples should had been performed to ensure that the decline of innate immunity in mucus was followed by an increase of those parameters in the plasma and in peritoneal cavity.

These hypothesis is in agreement with a work conducted with European seabass fed methionine diet, in which Machado et al. 2015 observed a stronger humoral and cellular response in both peripheral blood and inflammatory focus, after injection of *Phdp*. In the later study, an increased plasma peroxidase and lysozyme activity was reported, and higher plasma leucocytes counts registered, for fish fed

methionine supplemented diet, suggesting a lymphocyte migration to the inflammatory focus favored by methionine supplementation.

Several lines of evidence have reported the importance of skin as a peripheral immune organ in teleosts, due to not only the presence of bioactive molecules but also, due to the process of epidermal migration of leucocytes to the site of inflammation (Kania, Evensen, Larsen, & Buchmann, 2010). During an infection, a deviation of metabolic energy from peripheral tissue to the inflammatory focus is typically observed. Upregulation events normally take place during the initial response, generating pro-inflammatory molecules which contribute to a fast neutrophils recruitment (Esteban 2012). The role of fish skin as a source of these molecules has been confirmed (Gonzalez, Buchmann, & Nielsen, 2007), and in 2012 Lü et al. 2012 showed high expression of genes involved in leucocyte mobilization in zebra fish skin infected with the bacterium *Citrobacter freundii.* Together, these reports support that reduction of mucus immunity factors is being caused by the influx of neutrophils into the peritoneum via circulation, enhanced by methionine.

Trial 2- EFFECTS OF ARGININE AND CITRULLINE AVAILABILITY ON EUROPEAN SEABASS IMMUNE CONDITION

During the last decade, several studies have been focused in arginine application in functional diets, given its modulatory potential of both innate and adaptive immune systems in fish. Although efforts were made, the results obtained were often controversy and the fully mechanism of arginine immune-modulation is not yet understood. In the present work, fish were fed diets supplemented with arginine and citrulline (an arginine precursor) levels above the requirement for normal growth for European seabass in a feeding trial basis (i.e. 2 and 4 weeks), as well as following stimulation with bacteria. While the effects of arginine-enriched diet on plasma innate immunity has been reported for this species (Azeredo et al., 2015), this is the first work showing the modulatory effects of arginine and citrulline supplementation on the skin mucus immunity in European seabass.

The results of feeding trial revealed a beneficial effect upon arginine increment in peroxidase and bactericidal activities. Although not in a significant fashion,

supplementation of 0.5 and 1 % of ARG appears to improve mucus bactericidal capacity during the feeding trial, whereas no changes regarding this parameter were observed for fish fed the control diet during the trial period (4 weeks). The arginine´s immune stimulating effect suggested, is congruent with the results obtained for peroxidase values. Mucosal peroxidase values presented a clear tendency to rise over time in all supplemented groups, in contrast with fish fed the CTRL diet, which followed the opposite trend with a significant decline of peroxidase activity between 2 and 4 weeks. Arginine's positive influence in fish immune status could be related to polyamines biosynthesis. Polyamines, important products of arginine's metabolic pathway, are key molecules for leucocyte differentiation and proliferation (Andersen et al., 2013). As activated leucocytes release bioactive molecules, dietary arginine surplus may have benefited fish with extra humoral defences to better cope with an invading bacterium. This hypothesis is consistent with previous works where arginine supplementation was reported to increase leukocyte counts of juvenile Jian carp (*Cyprinus carpio var. Jian*) (G. Chen et al., 2015) and to stimulate the proliferation of channel catfish B lymphocytes in vitro (Pohlenz, Buentello, Mwangi, et al., 2012).

Under an infection condition in the present study, when bacterium was injected into peritoneal cavity, peroxidase levels were found to decrease over time, in all dietary groups. The declining peroxidase activity, independently of diet are probably a reflection of an obligatory neutrophil migration, a characteristic situation during the acute phase of an inflammatory response. This results suggest that the mucosal immune parameters are being deviated towards systemic immunity, regardless of nutritional stimuli.

In contrast, lysozyme levels behaved differently upon dietary administration. In all supplemented groups, a sharp reduction of these parameter was observed between 4 and 24 hours after infection with bacteria. However, fish fed dietary amino acids surplus showed a recovery in those levels at 48 hours following injection, as lysozyme activity improved and exceed values observed at 4 h following infection. The lysozyme response in supplemented fish stands out by its recuperation comparing, to what is observed in control group, where the enzyme's concentration kept declining alongside the development of the infection. The differences observed between the concentration of mucosal peroxidase and lysozyme activities in the time course trial, may rely on the source of these two enzymes. In fact, fish lysozyme may occur in various leukocytes, such as

neutrophils, monocytes and macrophages (Saurabh & Sahoo, 2008); unlike peroxidase, which is exclusively produced by neutrophils. The minimum lysozyme values observed at 24 hours, corresponding to the peak of the inflammatory response, may be partly attributed to neutrophils migration in response to inflammatory stimuli. However, lysozyme production is reestablished at 48 hours in fish fed supplemented diets, despite the neutrophils recruitment to inflammatory focus. This is probably due to activated macrophages present in the skin mucus, fueled by arginine (Saurabh & Sahoo, 2008). This hypothesis is consistent with previous works reporting that arginine levels are related to macrophages activity (Pohlenz, Buentello, Mwangi, et al., 2012) (Q. Zhou et al., 2014). Being a peripheral immune organ, far from the infection site, it possible that after 48 hours the skin mucus may have achieved restoration of lysozyme's activity more efficiently under arginine's supplementation. However, more studies are needed to confirm this pattern. Additionally, in future assays more sampling times should be performed after a bacterial insult, to follow both systemic and mucosal immune progression. Especially in the cases where the pathogen is activated, since fish requires more than 48h to solve the infection.

Simultaneously to mucus sampling, it would had been interesting to monitor plasma and peritoneal immune parameters to infer about leucocytes migrations, which would allow a better understanding of the evolution of the infection at a local level.

The overall results in both trials showed that mucus sampling is not reliable biomarker to distinguish between normal or disease status, as it does not clearly translate fish internal condition. The hematological information provided by blood samples cannot be replaced, however, as peripheral immune organ, great variation on mucus humoral profile may indicate a depressed immune status. Therefore, mucus sampling methods can be used as a routine procedure in aquaculture industries as it is easy, non-evasive and stress-free technique.

Probably, in the presence of an external infection, the mucus response would had been much more intense, consequently the supplementation effect much more visible.

Conclusions

The supplementation level of the studied AA was not enough to allow a strong influence in such a far peripheral tissue, as the skin mucus.

In this present study, no major variations in the skin mucus immunity in response to a peritoneal inflammation are detectable upon both dietary supplementations. However, given the overall results, the AA studied appear to divert peripheral metabolic energy towards the infectious site. Likewise, in the case of a bacteria whose route of infection was external, the previously described recruitment mechanisms, would be possibly occurring on epidermis tissue. Indeed, it would be interesting to test whether arginine, citruline or methionine would promote an enhanced cellular recruitment during a bath infection with the same pathogen. If this hypothesis is confirmed, fish would benefit from the incorporation of this AA in functional feeds, for periods previously known to be more susceptible to an outbreak of any skin infection.

References

Andersen, S. M., Holen, E., Aksnes, A., Rønnestad, I., Zerrahn, J.-E., & Espe, M. (2013). Dietary arginine affects energy metabolism through polyamine turnover in juvenile Atlantic salmon (Salmo salar). *The British Journal of Nutrition*, *110*(11), 1968–77. https://doi.org/10.1017/S0007114513001402

Andersen, S. M., Waagbø, R., & Espe, M. (2015). Functional amino acids in fish nutrition, health and welfare. *Frontiers in Bioscience (Elite Edition)*, *8*(1), 143–169.

Ángeles Esteban, M. (2012). An Overview of the Immunological Defenses in Fish Skin. *ISRN Immunology*, *2012*, 1–29. https://doi.org/10.5402/2012/853470

Azeredo, R., Pérez-Sánchez, J., Sitjà-Bobadilla, A., Fouz, B., Tort, L., Aragão, C., … Costas, B. (2015). European sea bass (Dicentrarchus labrax) immune status and disease resistance are impaired by Arginine dietary supplementation. *PLoS ONE*, *10*(10), 1–19. https://doi.org/10.1371/journal.pone.0139967

Basurco, B. (2000). Oddshore Mariculture in Mediterranean Contries. *Options Méditerranéennes Série B. Zaragoza, Spain.*, 9–18.

Batista, M. A., Nicoli, J. R., dos Santos Martins, F., Nogueira Machado, J. A., Esteves Arantes, R. M., Pacífico Quirino, I. E., … Cardoso, V. N. (2011). Pretreatment With Citrulline Improves Gut Barrier After Intestinal Obstruction in Mice. *Journal of Parenteral and Enteral Nutrition*, *36*(1), 69–76. https://doi.org/10.1177/0148607111414024

Breuillard, C., Bonhomme, S., Couderc, R., Cynober, L., & De Bandt, J.-P. (2014). In vitro anti-inflammatory effects of citrulline on peritoneal macrophages in Zucker diabetic fatty rats. *The British Journal of Nutrition*, (2015), 1–5. https://doi.org/10.1017/S0007114514002086

Buentello, J. A., & Gatlin, D. M. (2001). Effects of elevated dietary arginine on resistance of channel catfish to exposure to Edwardsiella ictaluri. *Journal of Aquatic Animal Health*, *13*(3), 194–201. https://doi.org/10.1577/1548-8667(2001)013<0194:EOEDAO>2.0.CO;2

Buentello, J. A., Reyes-Becerril, M., Romero-Geraldo, M. D. J., & Ascencio-Valle, F. D. J. (2007). Effects of dietary arginine on hematological parameters and innate immune function of channel catfish. *Journal of Aquatic Animal Health*, *19*(3),

195–203. https://doi.org/10.1577/H07-004.1

Buentello, J. a, & Gatlin Iii, D. M. (1999). Nitric oxide production in activated macrophages from channel catfish (Ictalurus punctatus): influence of dietary arginine and culture media. *Aquaculture*, *179*(1–4), 513–521. https://doi.org/10.1016/S0044-8486(99)00184-2

Chen, G., Liu, Y., Jiang, J., Jiang, W., Kuang, S., Tang, L., … Feng, L. (2015). Effect of dietary arginine on the immune response and gene expression in head kidney and spleen following infection of Jian carp with Aeromonas hydrophila. *Fish and Shellfish Immunology*, *44*(1), 195–202. https://doi.org/10.1016/j.fsi.2015.02.027

Chen, Q., Zhao, H., Huang, Y., Cao, J., Wang, G., Sun, Y., & Li, Y. (2016). Effects of dietary arginine levels on growth performance, body composition, serum biochemical indices and resistance ability against ammonia-nitrogen stress in juvenile yellow catfish (Pelteobagrus fulvidraco). *Animal Nutrition*, 1–6. https://doi.org/10.1016/j.aninu.2016.07.001

Cheng, Z., Buentello, A., & Gatlin, D. M. (2011). Effects of dietary arginine and glutamine on growth performance, immune responses and intestinal structure of red drum, Sciaenops ocellatus. *Aquaculture*, *319*(1–2), 247–252. https://doi.org/10.1016/j.aquaculture.2011.06.025

Cheng, Z., Gatlin, D. M., & Buentello, A. (2012). Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (Morone chrysops??Morone saxatilis). *Aquaculture*, *362–363*, 39–43. https://doi.org/10.1016/j.aquaculture.2012.07.015

Conceição, L. E. C., Aragão, C., Dias, J., Costas, B., Terova, G., Martins, C., & Tort, L. (2012). Dietary nitrogen and fish welfare. *Fish Physiology and Biochemistry*, *38*(1), 119–141. https://doi.org/10.1007/s10695-011-9592-y

Costas, B., Aragão, C., Soengas, J. L., Míguez, J. M., Rema, P., Dias, J., … Conceição, L. E. C. (2012). Effects of dietary amino acids and repeated handling on stress response and brain monoaminergic neurotransmitters in Senegalese sole (Solea senegalensis) juveniles. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *161*(1), 18–26. https://doi.org/10.1016/j.cbpa.2011.08.014

Costas, B., Conceição, L. E. C., Dias, J., Novoa, B., Figueras, A., & Afonso, A. (2011). Dietary arginine and repeated handling increase disease resistance and

modulate innate immune mechanisms of Senegalese sole (Solea senegalensis Kaup, 1858). *Fish and Shellfish Immunology*, *31*(6), 838–847. https://doi.org/10.1016/j.fsi.2011.07.024

Costas, B., Rêgo, P. C. N. P., Conceição, L. E. C., Dias, J., & Afonso, A. (2013). Dietary arginine supplementation decreases plasma cortisol levels and modulates immune mechanisms in chronically stressed turbot (Scophthalmus maximus). *Aquaculture Nutrition*, *19*(SUPL.1), 25–38. https://doi.org/10.1111/anu.12086

do Vale, A., Costa-Ramos, C., Silva, A., Silva, D. S. P., Gärtner, F., dos Santos, N. M. S., & Silva, M. T. (2007). Systemic macrophage and neutrophil destruction by secondary necrosis induced by a bacterial exotoxin in a Gram-negative septicaemia. *Cellular Microbiology*, *9*(4), 988–1003. https://doi.org/10.1111/j.1462-5822.2006.00846.x

Do Vale, A., Marques, F., & Silva, M. T. (2003). Apoptosis of sea bass (Dicentrarchus labrax L.) neutrophils and macrophages induced by experimental infection with Photobacterium damselae subsp. piscicida. *Fish and Shellfish Immunology*, *15*(2), 129–144. https://doi.org/10.1016/S1050-4648(02)00144-4

Do Vale, A., Silva, M. T., Dos Santos, N. M. S., Nascimento, D. S., Reis-Rodrigues, P., Costa-Ramos, C., … Azevedo, J. E. (2005). AIP56, a novel plasmid-encoded virulence factor of Photobacterium damselae subsp. piscicida with apoptogenic activity against sea bass macrophages and neutrophils. *Molecular Microbiology*, *58*(4), 1025–1038. https://doi.org/10.1111/j.1365-2958.2005.04893.x

Ellis, A. E. (1990). Serum antiproteases in fish. *Techniques in Fish Immunology*.

Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Developmental & Comparative Immunology*, *25*(8), 827–839. https://doi.org/https://doi.org/10.1016/S0145-305X(01)00038-6

FAO. (2012). Dicentrarchus labrax, 1–12.

FAO. (2016). *The State of World Fisheries and Aquaculture 2016*. Contributing to food security and nutrition for all. Rome.

Feng, F., Qing-hui, A., Wei, X., Kang-sen, M., & Wen-bing, Z. (2011). *Effects of dietary arginine and lysine on growth and non-specific immune responses of juvenile darkbarbel catfish (Pelteobagrus vachelli)*. Ocean University of China.

Gonzalez, S. F., Buchmann, K., & Nielsen, M. E. (2007). Real-time gene expression

analysis in carp (Cyprinus carpio L.) skin: Inflammatory responses caused by the ectoparasite Ichthyophthirius multifiliis. *Fish and Shellfish Immunology*, *22*(6), 641–650. https://doi.org/10.1016/j.fsi.2006.08.011

Grimble, R. F., Grimble, G. K., Poshoi, L., al., et, & Lauterberg, B. H. (1996). Immunonutrition: role of sulfur amino acids, related amino acids, and polyamines. *Nutrition (Burbank, Los Angeles County, Calif.)*, *14*(7–8), 605–10. https://doi.org/10.1016/S0899-9007(98)80041-5

Jiang, J., Shi, D., Zhou, X. Q., Hu, Y., Feng, L., Liu, Y., … Zhao, Y. (2015). Invitro and invivo protective effect of arginine against lipopolysaccharide induced inflammatory response in the intestine of juvenile Jian carp (Cyprinus carpio var. Jian). *Fish and Shellfish Immunology*, *42*(2), 457–464. https://doi.org/10.1016/j.fsi.2014.11.030

Kania, P. W., Evensen, O., Larsen, T. B., & Buchmann, K. (2010). Molecular and immunohistochemical studies on epidermal responses in Atlantic salmon Salmo salar L. induced by Gyrodactylus salaris Malmberg, 1957. *Journal of Helminthology*, *84*(2), 166–172. https://doi.org/10.1017/S0022149X09990460

Kaushik, S. (1998). Whole body amino acid composition of European seabass (Dicentrarchus labrax), gilthead seabream (Sparus aurata) and turbot { Psetta maxima) with an estimation of their IAA requirement profiles, *11*(5), 355–358.

Kiron, V. (2012). Fish immune system and its nutritional modulation for preventive health care. *Animal Feed Science and Technology*, *173*(1–2), 111–133. https://doi.org/10.1016/j.anifeedsci.2011.12.015

Kuang, S. Y., Xiao, W. W., Feng, L., Liu, Y., Jiang, J., Jiang, W. D., … Zhou, X. Q. (2012). Effects of graded levels of dietary methionine hydroxy analogue on immune response and antioxidant status of immune organs in juvenile Jian carp (Cyprinus carpio var. Jian). *Fish and Shellfish Immunology*, *32*(5), 629–636. https://doi.org/10.1016/j.fsi.2011.12.012

Li, P., Mai, K., Trushenski, J., & Wu, G. (2009). New developments in fish amino acid nutrition: Towards functional and environmentally oriented aquafeeds. *Amino Acids*, *37*(1), 43–53. https://doi.org/10.1007/s00726-008-0171-1

Li, P., Yin, Y.-L., Li, D., Kim, S. W., & Wu, G. (2007). Amino acids and immune function. *The British Journal of Nutrition*, *98*(2), 237–252. https://doi.org/10.1017/S000711450769936X

Lin, H., Tan, X., Zhou, C., Niu, J., Xia, D., Huang, Z., … Wang, Y. (2015). Effect of

dietary arginine levels on the growth performance, feed utilization, non-specific immune response and disease resistance of juvenile golden pompano Trachinotus ovatus. *Aquaculture*, *437*, 382–389. https://doi.org/10.1016/j.aquaculture.2014.12.025

Lopes, A. S. de C. (2016). From consumption to production – development of fisheries and aquaculture in Portugal. Retrieved from http://run.unl.pt/handle/10362/19237

Lü, A., Hu, X., Xue, J., Zhu, J., Wang, Y., & Zhou, G. (2012). Gene expression profiling in the skin of zebrafish infected with Citrobacter freundii. *Fish and Shellfish Immunology*, *32*(2), 273–283. https://doi.org/10.1016/j.fsi.2011.11.016

Machado, M., Azeredo, R., D??az-Rosales, P., Afonso, A., Peres, H., Oliva-Teles, A., & Costas, B. (2015). Dietary tryptophan and methionine as modulators of European seabass (Dicentrarchus labrax) immune status and inflammatory response. *Fish and Shellfish Immunology*, *42*(2), 353–362. https://doi.org/10.1016/j.fsi.2014.11.024

Magarinos, B., Toranzo, A. E., & Romalde, J. L. (1997). Phenotypic and Pathobiological, *6*(1), 41–64.

Métayer, S., Seiliez, I., Collin, A., Duchêne, S., Mercier, Y., Geraert, P.-A., & Tesseraud, S. (2008). Mechanisms through which sulfur amino acids control protein metabolism and oxidative status. *The Journal of Nutritional Biochemistry*, *19*(4), 207–15. https://doi.org/10.1016/j.jnutbio.2007.05.006

Mommsen, T., Moon, T., & Plisetskaya, E. (2001). Effects of arginine on pancreatic hormones and hepatic metabolism in rainbow trout. *Physiol Biochem Zool*, *74*, 668–678.

Moretti, A., Fernadez-Criado, M. P., Cittolin, G., & Guidastri, R. (1999). Manual on hatchery production of sea sass and gilthead seabream. *Fao*, *1*, 194.

Murray, C. K., & Fletcher, T. C. (1976). The immunohistochemical localization of lysozyme in plaice (Pleuronectes platessa L.) tissues. *Journal of Fish Biology*, *9*(4), 329–334. https://doi.org/10.1111/j.1095-8649.1976.tb04681.x

Nieves, C., & Langkamp-Henken, B. (2002). Arginine and immunity: A unique perspective. *Biomedicine and Pharmacotherapy*, *56*(10), 471–482. https://doi.org/10.1016/S0753-3322(02)00291-3

Pickering, A. D. (1974). The distribution of mucous cells in the epidermis of the brown trout Salmo trutta (L.) and the char Salvelinus alpinus (L.). *Journal of Fish Biology*, *6*(2), 111–118. https://doi.org/10.1111/j.1095-

8649.1974.tb04531.x

Pohlenz, C., Buentello, A., Criscitiello, M. F., Mwangi, W., Smith, R., & Gatlin, D. M. (2012). Synergies between vaccination and dietary arginine and glutamine supplementation improve the immune response of channel catfish against Edwardsiella ictaluri. *Fish and Shellfish Immunology*, *33*(3), 543–551. https://doi.org/10.1016/j.fsi.2012.06.005

Pohlenz, C., Buentello, A., Mwangi, W., & Gatlin, D. M. (2012). Arginine and glutamine supplementation to culture media improves the performance of various channel catfish immune cells. *Fish & Shellfish Immunology*, *32*(5), 762–768. https://doi.org/10.1016/j.fsi.2012.01.029

Pohlenz, C., & Gatlin, D. M. (2014). Interrelationships between fish nutrition and health. *Aquaculture*, *431*, 111–117. https://doi.org/10.1016/j.aquaculture.2014.02.008

Quade, M. J., & Roth, J. A. (1997). A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Veterinary Immunology and Immunopathology*, *58*(3), 239–248. https://doi.org/http://dx.doi.org/10.1016/S0165-2427(97)00048-2

Ramalho, A., Dinis, M. T., & Ccmar-cimar LA. (2011). Portuguese aquaculture : Current status and future perspectives. *World Aquaculture*, *42*(1), 26–32.

Ren, M., Liao, Y., Xie, J., Liu, B., Zhou, Q., Ge, X., … Chen, R. (2013). Dietary arginine requirement of juvenile blunt snout bream, Megalobrama amblycephala. *Aquaculture*, *414–415*, 229–234. https://doi.org/10.1016/j.aquaculture.2013.08.021

Romalde, J. L. (2002). Photobacterium damselae subsp. piscicida: An integrated view of a bacterial fish pathogen. *International Microbiology*, *5*(1), 3–9. https://doi.org/10.1007/s10123-002-0051-6

Rubin, L. L., Canal, C. W., Ribeiro, A. L. M., Kessler, A., Silva, I., Trevizan, L., … Kras, R. (2007). Effects of Methionine and Arginine Dietary Levels on the Immunity of Broiler Chickens Submitted to Immunological Stimuli. *Brazilian Journal of Poultry Science*, *9*(4), 241–247. https://doi.org/10.1590/S1516-635X2007000400006

Salinas, I. (2015). The Mucosal Immune System of Teleost Fish. *Biology*, *4*(3), 525–39. https://doi.org/10.3390/biology4030525

Salinas, I., Zhang, Y. A., & Sunyer, J. O. (2011). Mucosal immunoglobulins and B cells of teleost fish. *Developmental and Comparative Immunology*, *35*(12),

1346–1365. https://doi.org/10.1016/j.dci.2011.11.009

Saurabh, S., & Sahoo, P. K. (2008). Lysozyme: An important defence molecule of fish innate immune system. *Aquaculture Research*, *39*(3), 223–239. https://doi.org/10.1111/j.1365-2109.2007.01883.x

Shephard, K. L. (1994). Functions for fish mucus. *Reviews in Fish Biology and Fisheries*, *4*(4), 401–429. https://doi.org/10.1007/BF00042888

Stevens, M. G., Kehrli, M. E., & Canning, P. C. (1991). A colorimetric assay for quantitating bovine neutrophil bactericidal activity. *Veterinary Immunology and Immunopathology*, *28*(1), 45–56. https://doi.org/10.1016/0165-2427(91)90042-B

Subramanian, S., MacKinnon, S. L., & Ross, N. W. (2007). A comparative study on innate immune parameters in the epidermal mucus of various fish species. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, *148*(3), 256–263. https://doi.org/10.1016/j.cbpb.2007.06.003

Sunyer, J. O., & Tort, L. (1995). Natural hemolytic and bactericidal activities of sea bream Sparus aurata serum are effected by the alternative complement pathway. *Veterinary Immunology and Immunopathology*, *45*(3–4), 333–345.

Tang, L., Wang, G. X., Jiang, J., Feng, L., Yang, L., Li, S. H., … Zhou, X. Q. (2009). Effect of methionine on intestinal enzymes activities, microflora and humoral immune of juvenile Jian carp (cyprinus carpio var. Jian). *Aquaculture Nutrition*, *15*(5), 477–483. https://doi.org/10.1111/j.1365-2095.2008.00613.x

Wang, W. W., Qiao, S. Y., & Li, D. F. (2009). Amino acids and gut function. *Amino Acids*, *37*(1), 105–110. https://doi.org/10.1007/s00726-008-0152-4

Wu, G., Bazer, F. W., Davis, T. A., Kim, S. W., Li, P., Marc Rhoads, J., … Yin, Y. (2009). Arginine metabolism and nutrition in growth, health and disease. *Amino Acids*, *37*(1), 153–168. https://doi.org/10.1007/s00726-008-0210-y

Xie, F., Zeng, W., Zhou, Q., Wang, H., Wang, T., Zheng, C., & Wang, Y. (2012). Dietary lysine requirement of juvenile Pacific white shrimp, Litopenaeus vannamei. *Aquaculture*, *358–359*, 116–121. https://doi.org/10.1016/j.aquaculture.2012.06.027

Zaccone, G., Kapoor, B. G., Fasulo, S., & Ainis, L. (2001). Structural, histochemical and functional aspects of the epidermis of fishes. In B. T.-A. in M. Biology (Ed.) (Vol. Volume 40, pp. 253–348). Academic Press. https://doi.org/http://dx.doi.org/10.1016/S0065-2881(01)40004-6

Zhang, K., Mai, K., Xu, W., Liufu, Z., Zhang, Y., Peng, M., … Ai, Q. (2017). Effects of

dietary arginine and glutamine on growth performance , nonspeci fi c immunity , and disease resistance in relation to arginine catabolism in juvenile turbot (Scophthalmus maximus L .). *Aquaculture*, *468*, 246–254. https://doi.org/10.1016/j.aquaculture.2016.10.021

Zhou, H., Chen, N., Qiu, X., Zhao, M., & Jin, L. (2012). Arginine requirement and effect of arginine intake on immunity in largemouth bass, Micropterus salmoides. *Aquaculture Nutrition*, *18*(1), 107–116. https://doi.org/10.1111/j.1365-2095.2011.00886.x

Zhou, Q. C., Zeng, W. P., Wang, H. L., Xie, F. J., & Zheng, C. Q. (2012). Dietary arginine requirement of juvenile yellow grouper Epinephelus awoara. *Aquaculture*, *350–353*, 175–182.

Zhou, Q., Jin, M., Elmada, Z. C., Liang, X., & Mai, K. (2014). Growth, immune response and resistance to Aeromonas hydrophila of juvenile yellow catfish, Pelteobagrus fulvidraco, fed diets with different arginine levels. *Aquaculturefile:///Users/MariaCarlota/Documents/Mestrado/Tese/artigos novos/1-s2.0-S0044848614006486-Main.pdf file:///Users/MariaCarlota/Documents/Mestrado/Tese/artigos novos/1-s2.0-S0044848616307736-Main.pdf file:///Users/MariaCarlota/Documents/Mestrado/*, *437*, 84–91. https://doi.org/10.1016/j.aquaculture.2014.11.030